

GENETIC REGULATION OF FORMATE HYDROGENLYASE IN *ESCHERICHIA COLI*

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1991

ACKNOWLEDGEMENTS

I would like to sincerely thank my advising professor Dr. K. T. Shanmugam. His continual encouragement, guidance and enthusiasm for science have definitely influenced me for a lifetime. I would like to thank the K. T. S. laboratory group including both old and new. I would also like to extend my thanks to Drs. Richard Boyce, Francis Davis Jr., John Gander, and Lonnie Ingram for their helpful questions and counsel while serving on my committee. Additionally, I would like to thank Drs. A. Böck and B. Bachmann for providing several strains and plasmids necessary for this study. Finally, special thanks are extended to my family and fiancé for their willingness to listen to my ups and downs throughout the course of this work.

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LIST OF ABBREVIATIONS

A	Absorbance
Ac-CoA.	Acetyl coenzyme A
ATP	Adenosine triphosphate
BV	Benzyl viologen
CAT	Chloramphenicol acetyltransferase
CRP	cAMP repressor protein
CTAB	Hexadecyltrimethylammonium bromide
DMS	Dimethyl sulfide
DMSO.	Dimethyl sulfoxide
FDH-H	Formate dehydrogenase (linked to H ₂ evolution)
FDH-N	Formate dehydrogenase (linked to NO ₃ ⁻ reduction)
FHL	Formate hydrogenlyase
FR.	Fumarate reductase
HUP	Hydrogen uptake activity
HYD	Hydrogenase
HYD-1	Hydrogenase isoenzyme-1
HYD-2	Hydrogenase isoenzyme-2
HYD-3	Hydrogenase isoenzyme-3
LB.	Luria Broth

LBG	Luria Broth + glucose
LBGF.	Luria Broth + glucose + formate
LBF	Luria Broth + formate
LBM	Luria Broth + maltose
LBN	Luria Broth + nitrate
LBNF.	Luria Broth + nitrate + formate
MES	2-(N-Morpholino)ethanesulfonic acid
Mo.	Molybdenum
MOPS.	3-(N-Morpholino)propanesulfonic acid
MPT	Molybdopterin
MGD	Molybdopterin guanine dinucleotide
NAD ⁺ , NADH.	Nicotinamide adenine dinucleotide and its reduced form, respectively
OAA	Oxaloacetate
ONPG	Ortho-nitrophenyl- β -D-galactopyranoside
ORF	Open reading frame
PDH	Pyruvate dehydrogenase
PEP	Phosphoenolpyruvate
PFL	Pyruvate formate lyase
PIPES	Piperazine-N,N'-bis[2-ethanesulfonic acid]; 1,4-piperazinediethanesulfonic acid
PMF	Proton motive force
SDS	Sodium dodecyl sulfate
TES	N-Tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid
TMA	Trimethylamine

TMAO.	Trimethylamine-N-oxide
Tris.	Tris-(hydroxymethyl)-aminomethane
X-gal	5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside

LIST OF GENE SYMBOLS

All the genes listed below are from *Escherichia coli* unless otherwise indicated.

<u>Gene symbol</u>	<u>Alternate gene symbols; phenotype affected</u>
<i>ant</i>	See <i>hyc</i> ; <u>anaerobic electron transport</u>
<i>arcA</i>	<u>Aerobic regulatory control</u> , putative DNA binding protein of Arc modulon
<i>arcB</i>	<u>Aerobic regulatory control</u> , histidine-protein-kinase of Arc modulon
<i>chlA</i>	Synthesis of the pterin component of MPT
<i>chlB</i>	"Association factor-F _A "; synthesis of functional MPT
<i>chlC</i>	See <i>narGHJI</i>
<i>chlD</i>	Peripheral protein of molybdate binding-protein-dependent transport system
<i>chlE</i>	Synthesis of the pterin component of MPT
<i>chlF</i>	See <i>fdhGHI</i>
<i>chlG</i>	Molybdate-restorable nitrate reductase activity
<i>chlJ'</i>	Integral membrane protein of molybdate binding-protein-dependent transport
<i>cyd</i>	Cytochrome <i>d</i> ; high-affinity oxidase
<i>cyo</i>	Cytochrome <i>o</i> ; low-affinity oxidase
<i>dctD</i>	C-4 dicarboxylate transport; <i>Rhizobium leguminosarum</i>
<i>dmsABC</i>	DMSO and TMAO reductase activity

<i>ebgA</i>	Ancestral gene for β -galactosidase
<i>fdhF</i>	FDH-H; formate dehydrogenase (linked to H_2 evolution)
<i>fdhGHI</i>	FDH-N; formate dehydrogenase (linked to NO_3^- reduction)
<i>fhlA</i>	Putative DNA binding protein necessary for transcriptional activation of the <i>fdhF</i> , <i>hyc</i> and <i>fhlB</i> operons
<i>fhlB</i>	Putative modulator of the FHL pathway; necessary for FHL, FDH-H and total HYD activity
<i>flbD</i>	Flagellar synthesis; <i>Caulobacter crescentus</i>
<i>fnr</i>	Global regulator of anaerobic respiration; homologous to CRP
<i>frdABCD</i>	Fumarate reductase
<i>gyrB</i>	DNA gyrase
<i>his</i>	Histidine biosynthesis and binding-protein-dependent transport system
<i>hyb</i>	Hydrogenase isoenzyme-2
<i>hyc</i>	Hydrogenase isoenzyme-3
<i>hydA</i>	Total hydrogenase activity
<i>hydFB</i>	<i>hypDE</i> ; total hydrogenase activity
<i>hydE</i>	<i>hypB</i> ; total hydrogenase activity; Ni suppressible
<i>hydC,D</i>	Total hydrogenase activity; nickel transport
<i>hyd-17</i>	ORF5 of the <i>hyc</i> operon, hydrogenase isoenzyme-3
<i>hydG</i>	Putative DNA binding protein of dihydrogen metabolism (uptake)
<i>hyp</i>	Total hydrogenase activity, operon includes previously described <i>hydB</i> , <i>E</i> , and <i>F</i> genes
<i>mal</i>	Maltose metabolism and binding-protein-dependent transport system
<i>molR</i>	Molybdate binding-protein-dependent transport system

<i>narL</i>	Putative DNA binding protein of nitrate regulation
<i>narX</i>	Putative membrane-bound histidine-protein-kinase of nitrate regulation
<i>narQ</i>	Second proposed histidine-protein-kinase of nitrate regulation
<i>narGHJI</i>	Nitrate reductase
<i>nifA</i>	Positive activator for nitrogen fixation; <i>Klebsiella pneumoniae</i>
<i>ntrC</i>	<i>glnG</i> , nitrogen metabolism
<i>pfl</i>	Pyruvate formate lyase
<i>pgi</i>	<i>oxrC</i> , phosphoglucose isomerase
<i>tyrR</i>	Aromatic amino acid biosynthesis and transport
<i>xylR</i>	Degradative pathway of aromatic hydrocarbons; <i>Pseudomonas putida</i>

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

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December, 1991

Chairman: Dr. K. T. Shanmugam

Major Department: Microbiology and Cell Science

A new gene (*fhlB*) whose product regulates the production of formate hydrogenlyase (FHL) has been identified in *Escherichia coli*. Biochemical analysis of a mutant, strain SE-2011 [$\Phi(fhlB'$ - $'lacZ^+$)], revealed that this mutant lacks formate dehydrogenase activity associated with FHL (FDH-H) and hydrogenase activity. As a consequence, dihydrogen production and uptake were undetectable in strain SE-2011. Expression of the *fhlB* gene (measured as β -galactosidase activity) was increased 2- to 3-fold by anaerobic conditions, and enhanced by formate, but only under anaerobic conditions. Maximum expression of $\Phi(fhlB'$ - $'lacZ^+$) required *rpoN*, *fhlA*, *chlD*, and *molR* gene products. The concentration of formate required for maximum expression of the *fhlB* gene was reduced in the presence of *fhlA* gene in a multicopy plasmid from about 15 mM to 3 mM. The *fhlB* gene in *molR* or *chlD* genetic

background still retained formate inducibility; however, molybdate was required at high concentrations for expression to wild type levels. DNA sequence analysis of *fhlA* gene revealed that the FHL-A protein is homologous to other known transcriptional activators. It is proposed that formate, in association with the FHL-A protein, initiates transcription of the *fhlB*, *hyc*, *hyp* and *fdhF* operons. Analysis of mutant strain SE-2007 [$\Phi(fhlA'-lacZ^+)$] indicated that the transcription of the *fhlA* gene, located at the 3'-end of the *hyp* operon, is constitutive. This is in contrast to the *hyp* operon transcription which has dual controls: formate-dependent and FNR-dependent. The DNA sequence of the *mol* operon (essential for molybdate transport, FHL activity and *fhlB* expression) was also determined to investigate the role of molybdate metabolism in the regulation of FHL in *E. coli*. These studies revealed that the fermentative dihydrogen production is regulated by a complex series of reactions involving the availability of formate, Mo and absence of O_2 . It is postulated that the FHL-B protein is one of the modulators of this control system.

INTRODUCTION

The production of dihydrogen by the members of the Enterobacteriaceae family has been known since 1901 (166). Although much has been learned since that time, many avenues of research remain to be explored. It is now understood that the formate hydrogenlyase complex is composed of FDH-H, HYD-3, and associated electron transport proteins (10, 27, 72, 173, 193, 215, 243). This complex catalyzes the oxidation of formate to H_2 and CO_2 (2, 166) and is found only in anaerobically growing cells. The reaction catalyzed by the FHL complex operates at a standard free energy ($\Delta G_0'$) of zero and does not appear to be involved in cellular energy production (2). It is presumed that the FHL system plays an important role in later stages of the microorganism's fermentative growth when organic acids such as formate accumulate and lower the pH of the culture medium. The cell apparently synthesizes the FHL complex in order to modulate the external pH, thus facilitating growth (77).

The FHL pathway is an attractive research field due to the potential of harnessing dihydrogen production as an alternative energy source. Current events, including the Persian Gulf War and concern over the greenhouse effect, have aroused the public's attention. Although lobbyists continue to solicit Congress for the oil industry, the

American population is more aware of this crisis and open to environmentally and economically sound options. Research in these fields must continue in order for alternative fuels to become more competitive. Unfortunately, historical events, i.e. the "Hindenberg", have curbed the use of dihydrogen as an energy source. When properly controlled, dihydrogen is a clean burning fuel. The end product being H_2O , instead of CO , CO_2 , SO_2 and nuclear waste (the current waste products of energy production in our country).

Additionally, the question of how an organism responds to various environmental stimuli such as dioxygen and regulates the metabolism by activating or repressing specific genes remains to be fully elucidated. By studying the FHL system of *Escherichia coli*, one can not only integrate regulatory similarities from this system to other pathways, but also study modes of control which are unique to FHL in order to gain a clear understanding of gene expression. Associating this with the physiological role of anaerobic H_2 and CO_2 production is also of interest. The metabolic fate of the end products of FHL are poorly understood.

Following the advent of DNA sequencing techniques and computer programs designed to organize the sequences generated, studies on protein homology have enabled the researcher to identify apparent function from the primary structure. It is now understood that there are two categories of proteins involved in signal transduction: sensor and receiver. Generally, the sensor is a membrane-bound, histidine-

protein-kinase. Appropriate environmental stimulation results in sensor autophosphorylation cascading to phosphorylation of the receiver protein. Once in an active form, the receiver is a positive regulator of specific modulon transcription (163, 183, 222).

Much remains to be understood about how the "appropriate environmental stimulation" is transmitted to the sensor protein. It has been proposed that the FHL system is regulated by five environmental signals which include anaerobiosis, intracellular formate, pH, molybdate, and nickel. Although the genes encoding the structural components of the FHL complex [*fdhF* (FDH-H) and *hyc* (HYD-3) operons] are located approximately 33 minutes apart in the *E. coli* chromosome, their transcription appears to be coordinated (11, 24, 25, 171).

In order to decipher the mode by which these signals are transmitted to the transcription of the formate modulon, a series of promoter *lac* fusion strains were constructed and characterized for anaerobic induction and dihydrogen related enzyme activities. Mutational analysis revealed a unique gene, later denoted as the *fhlB* gene, encoding a product essential for FHL activity including FDH-H and total hydrogenase activity. However, FHL-B protein was not necessary for expression of the *fdhF* gene and is therefore currently assumed modulate transcription of the FHL pathway (Fig. 1). Transcription of the *fhlB* gene was repressed by high-redox potential electron acceptors (dioxxygen and nitrate) and required formate for maximum anaerobic induction. Further analysis revealed that *rpoN* (encoding the σ^{54}

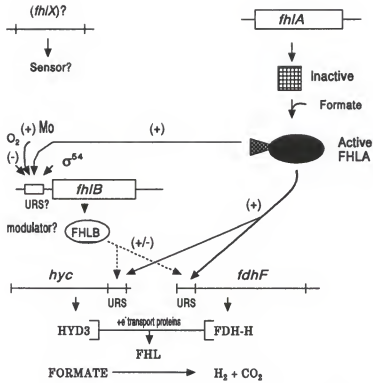


Figure 1. Model of genetic regulation of formate hydrogenlyase in *E. coli*.

subunit of RNA polymerase), *fhIA*, *chID* and *molR* gene products were necessary for optimum *fhIB* gene transcription (Fig. 1).

Subsequent investigation of the *fhIB* gene transcription demonstrated that high cytoplasmic concentrations of FHL-A protein (using multicopy plasmid pSE-133) decreased but did not eliminate the formate requirement. Because of these observations, the DNA sequence of the *fhIA* gene was determined to identify the characteristics of the gene and its product. The FHL-A protein was found to have sequence homology with putative σ^{54} -dependent transcriptional activators (receivers) of the two-component regulatory system. However, the amino terminus of the FHL-A protein was unique and did not contain the conserved secondary structure or aspartate and lysine residues which have been shown to be phosphorylated by the respective histidine-protein-kinase (sensor). This would suggest that phosphorylation is not required for active FHL-A protein, and a sensor protein may not be involved. Interestingly, transcription of the *fhIA* gene was shown to be from the weak "-35 and -10" *fhIA* promoter (constitutive) and not from the upstream FNR- or σ^{54} -dependent promoters of the *hyp* operon. It is therefore possible that the FHL-A protein is synthesized in an inactive form and activated in the presence of formate (Fig. 1).

During investigation of the molybdate requirement for FHL, it was determined that the *fhIB* gene in *molR* or *chID* genetic backgrounds still retained formate inducibility in the presence of multiple copies of the *fhIA*⁺ gene (exogenous molybdate was required for expression to wild type

levels). This indicates that FHL is regulated by a dual regulatory system, one involving molybdate and a second formate-dependent pathway (Fig. 1). The results presented show that fermentative dihydrogen production is regulated by a complex series of reactions involving the availability of formate, molybdate and the absence of oxygen.

LITERATURE REVIEW

Facultative anaerobes, such as *Escherichia coli*, have evolved respiratory networks organized such that the most energetically favorable electron transport pathway is utilized for the specific redox state of the environment. *E. coli* is capable of using a variety of electron acceptors which include in order of decreasing potential: $1/2O_2/H_2O$ ($E_o' = +0.82$ V), NO_3^-/NO_2^- ($E_o' = +0.42$ V), NO_2^-/NH_4^+ ($E_o' = +0.36$ V), $DMSO/DMS$ ($E_o' = +0.16$ V), $TMAO/TMA$ ($E_o' = +0.13$ V), fumarate/succinate ($E_o' = +0.03$ V), pyruvate/lactate ($E_o' = -0.19$ V), acetaldehyde/ethanol ($E_o' = -0.2$ V), H^+/H_2 ($E_o' = -0.42$ V; ref. 16, 228). Pathway preference is determined by the difference between the standard oxidation-reduction potential of the initial electron-donor (NADH) and terminal acceptor system ($\Delta E_o'$) available in the growth medium. The standard free energy change ($\Delta G_o'$) of a reaction is given by

$$\Delta G_o' = -nF\Delta E_o'$$

where n is the number of electrons transferred and F is Faraday's constant. Therefore the greater the $\Delta E_o'$, the more free energy can be harnessed by the organism for biosynthetic purposes.

This establishes a hierarchy with aerobic respiration being the most energetically favorable for the cell's survival. Thus, other

electron transport pathways are not synthesized in the presence of dioxygen. Furthermore when nitrate is present in an anaerobic environment, the nitrate respiratory pathway is induced and alternate respiratory systems are repressed (for review, see 82, 102, 139, 175). Presumably, a complex interconnected regulation involving transcription, translation, protein processing, and allosteric effectors is conceivable.

Respiration involves the generation of a proton motive force (PMF), consisting of a pH gradient (ΔpH) and electrochemical gradient ($\Delta\psi$), by electron transport (for review, see 84, 160). In general, respiratory components are organized into "modules" or pathways. These include a substrate dehydrogenase, which transfers electrons to a quinone pool, and ultimately a membrane-bound reductase; thus coupling substrate oxidation to the reduction of an electron acceptor (139). A fermenting cell, on the other hand, produces endogenous electron acceptors and derives most of its energy from substrate-level phosphorylation (42). Although it is generally believed that the FHL system is a fermentative process discrete from anaerobic respiration, the precise function of the FHL pathway remains unclear.

E. coli is a mixed-acid fermentor producing acetate, lactate, formate, ethanol, succinate, H_2 and CO_2 (Fig. 2). The ratio of these major end products is regulated (conceivably by redox-mediated modulation) to balance the reducing equivalents generated during glycolysis (3, 206). All of these fermentation products are derived

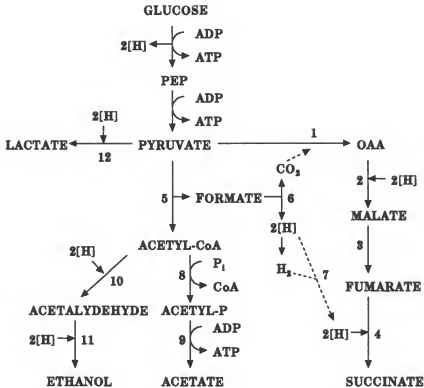


Figure 2. Mixed-acid fermentation of *E. coli*. 1, Phosphoenolpyruvate carboxylase; 2, malate dehydrogenase; 3, fumarase; 4, fumarate reductase; 5, pyruvate formate lyase; 6, formate hydrogen lyase complex (formate dehydrogenase-H, hydrogenase isoenzyme-3, and associated electron transport proteins); 7, hydrogenase isoenzyme-2; 8, phosphotransacetylase; 9, acetate kinase; 10, acetaldehyde dehydrogenase; 11, alcohol dehydrogenase; 12, lactate dehydrogenase. Redrawn with modifications from Alam and Clark (3).

from pyruvate. It has been proposed that PDH is inhibited by the high $[NADH]/[NAD^+]$ ratio reached upon shift to anaerobic conditions (83). Therefore anaerobically active PFL, encoded by the *pfl* operon, plays a central role in glucose metabolism (122, 170, 191). The PFL complex catalyzes the nonoxidative cleavage of pyruvate leading to transfer of "acetyl" group to coenzyme A (Ac-CoA) and formate (75). Consequently, the CO_2 and reduced NADH produced aerobically by PDH is replaced anaerobically by formate. This is critical since the cell must maintain an acceptable ratio of $[NADH]/[NAD^+]$ (42). The FHL catalyzes the oxidation of formate to H_2 and CO_2 . This minimizes the accumulation of formic acid which would otherwise lower the pH (77). The metabolic fates of the gaseous products, H_2 and CO_2 , are poorly understood. It is hypothesized that FHL is the major anaerobic enzyme producing CO_2 for the synthesis of oxaloacetate (OAA) from phosphoenol pyruvate (PEP; ref. 42). OAA can be further metabolized to succinate or 2-oxoglutarate, all three of these metabolites being essential for biosynthetic reactions such as amino acid production. *E. coli* can utilize the H_2 produced from formate oxidation by FHL to reduce fumarate to succinate (HUP; ref. 148). This energy yielding pathway is HYD-2 dependent (12, 151, 193). This would link FHL to the membrane-bound fumarate reductase and thus a respiratory process which provides the cell with 1 mole of ATP per mole of fumarate reduced, presumably by the generation of PMF (116). Therefore, FHL appears to be linked to both fermentative and respiratory pathways.

Formate hydrogenlyase is composed of FDH-H, HYD-3, and redox carriers linking the two enzymes (27, 77, 173, 193). The *fdhF* gene (92.4 min) encodes the 80 kDa selenopolypeptide constituent of FDH-H (11, 243). Additionally, the FDH-H protein requires molybdenum (molybdopterin-guanylate) for activity (33, 73, 112). The *hyc* operon (59 min) consists of 8 ORFs and is presumed to encode the remaining FHL components. Five of these ORFs code for electron carriers (previously termed *ant* and *hyd-17*; ref. 11, 27, 171, 242). The ORF5 (*hyd-17*) shows significant sequence homology with the large subunit from other Ni/Fe hydrogenases and is the presumptive structural gene for HYD-3.

Numerous mutants have been isolated with defects in H₂ metabolism (Fhl⁻), the majority of which have been deficient in all three hydrogenase isoenzymes (35, 37, 76, 107, 128, 135, 167, 171, 231). Through the characterization of mutant strains, DNA sequence analysis and mapping of the genes affected, FHL regulation can be more clearly understood. It is proposed that the FHL system is regulated by signals which include anaerobiosis, nitrate, intracellular formate, low pH, molybdate, nickel, and selenium. How these signals are transmitted to the transcription, translation and post-translational modification of the components of the FHL complex remains poorly understood.

Anaerobiosis

The presence or absence of terminal electron acceptors, such as dioxygen or nitrate, play a major role in regulating genes involved in respiration and fermentation. It was estimated, by using 2-dimensional

protein electrophoresis, that 18 or more proteins were induced by anaerobiosis in *E. coli* (205). Later studies using a physiological approach with *lac* gene fusions, estimated that 50 genes were induced when dioxygen and alternative electron acceptors were absent, 20% of these were H₂ repressible, and only a small number of mutants failed to grow microaerobically on glucose minimal medium (41).

The FHL pathway has been shown to be induced anaerobically by a variety of methods. Initially, FHL induction was monitored by an increase in specific activity of FHL and the enzymes of the pathway (10, 57, 78, 127, 203, 235, 236). Later, transcription of the *fdhF* and *hyc* (*hyd-17*) operons (structural genes of FHL), measured as β -galactosidase activity using gene fusion strains, was found to be anaerobically inducible (171). These studies demonstrate that anaerobically growing organisms require the synthesis of additional proteins to generate energy and maintain redox balance. Unfortunately, little is known about how the organism senses the onset of anaerobiosis and activates or represses specific pathways.

Many earlier studies have suggested that anaerobic electron transport systems are subject to a "dual control" in which an unidentified effector molecule somehow senses the "redox" status of the environment and activates FNR protein, a pleiotropic transcriptional activator and repressor. In combination with this global regulatory mechanism, specific transcriptional effectors responded to terminal electron acceptors such as nitrate or fumarate (36, 47, 197, 198). With

the identification and characterization of the Arc system and other forms of respiratory control, current research forms a much more complex picture of regulation (105, 106, 212).

Available evidence suggests that this aerobic-anaerobic switch may involve several genes including *arcA*, *arcB*, *fnr* and *pgi* (involved in DNA-supercoiling); mutants deficient in any one of these genes have pleiotropic phenotypes. The Arc modulon is a two-component sensor-regulator system (105, 106). Through DNA sequence analysis, ARC-B is presumed to be a membrane-bound, histidine-protein kinase. Autophosphorylation of ARC-B occurs in response to dioxygen-limitation (possibly redox control). The signal is then transmitted to ARC-A by phosphorylation to produce a transcriptional activator of the cytochrome *d* (a high-affinity oxidase) gene (*cyd*). ARC-A is also a repressor of "aerobic" enzyme synthesis (i.e. succinate dehydrogenase and cytochrome *o*; ref. 68, 105). Cytochrome *o*, having a lower affinity for dioxygen, is only synthesized aerobically; where as cytochrome *d* is stimulated under microaerobic conditions as the terminal electron carrier (for review, see 175).

The FNR protein, likewise, acts as both a transcriptional activator and repressor (for review, see 211, 212). Mutations in the *fnr* gene were originally isolated as strains deficient in nitrate reductase and fumarate reductase (133). It is now known that several other anaerobically inducible enzymes require FNR for transcription and include nitrite reductase (both *cyt*₅₅₂ and NADH-linked; ref. 38, 45,

159), DMSO:TMAO reductase (216, 232), PFL (190, 191), FDH-N (19, 25); anaerobic glycerol-3-phosphate dehydrogenase (130), fumarase B (18, 237), aspartase, and asparaginase II (109). The FNR protein represses its own synthesis (autoregulation; ref. 209) as well as cytochrome *o* (low-affinity oxidase; ref. 50, 68) and NADH dehydrogenase II (213). Recent evidence is conflicting on FNR regulation at the *cyd* promoter. By monitoring *cyd* expression at varying levels of dissolved dioxygen concentrations, Fu *et al.* (68) suggest that FNR, a weaker transcriptional activator at this operon, outcompetes ARC-A for operator binding as the culture shifts from microaerobic to anaerobic conditions.

The FNR protein has been shown through sequence analysis to be homologous to cAMP receptor protein (CRP) in the helix-turn-helix and nucleotide binding domains (199). However, the FNR amino-terminus differs. It is cysteine rich and three of the four cysteine residues are proposed to be involved in redox-sensitive iron-binding (210). The addition of ferrozine, an iron specific chelator, to the growth medium reduced the level of transcription of the *frd* operon (encoding fumarate reductase). Since this operon is positively regulated by FNR, iron in association with FNR may be involved in this regulation. In support of this possibility, Green *et al.* (79) purified FNR in the presence of externally added iron and demonstrated specific *in vitro* binding of the pure protein to a synthetic (postulated) FNR-binding site by both DNaseI and methylation-protection (DMS) footprinting. Although direct evidence is still lacking, parallels can be drawn with the thoroughly

investigated, metal-dependent regulation of FUR (iron uptake) and MER-R (mercury resistance) systems (for review, see 90). It is possible that molybdenum and nickel (necessary for transcriptional repression of *chlD* and *hydC* genes, respectively) be also involve a metal-protein complex.

Formate hydrogenlyase synthesis is independent of the ARC system, and FNR-dependence appears to be indirect (44, 105, 171). The transcription of the *pfl* gene which is essential for the production of formate is partially FNR dependent (190, 191). The gene coding for nickel transport (*hydC*) is also FNR dependent (239). The *hyp* operon, whose products presumably process the nickel into forms suitable for hydrogenase, appears to be FNR regulated when the intracellular formate concentration is limiting (this study).

DNA supercoiling has been suggested to be essential for transcription from several promoters including the FNR-independent promoters of the FHL pathway. Early studies have shown that mutants lacking the DNA gyrase activity are also impaired in anaerobic growth (241). Additionally, FHL activity is absent and plasmid supercoiling is altered in a glucose-grown *pgi* (*oxrC*) mutant. Phosphoglucose isomerase, a glycolytic enzyme encoded by the *pgi* (*oxrC*) gene, is necessary for the synthesis of FDH-H, peptidase T, tripeptide permease, HYD-1 and HYD-3 in *Salmonella typhimurium* (107, 108). Recently, Hsieh *et al.* (98) have compared changes in [ATP]/[ADP] ratios and negative supercoiling of both chromosomal and plasmid DNA upon shifting *E. coli* from aerobic to anaerobic conditions. They monitored the effect of dioxygen tension on

supercoiling activity in wild type and *gyrB* (DNA gyrase) mutants. It was concluded that the respiratory state of the organism influenced DNA supercoiling. Furthermore, this was mediated by DNA gyrase since, changes in supercoiling paralleled the [ATP]/[ADP] ratios. This is analogous to *in vitro* studies which demonstrated that the [ATP]/[ADP] ratio influences gyrase-mediated DNA supercoiling (233). Therefore, the energy status of the organism may be an environmental signal affecting DNA supercoiling. In general, the state of chromosomal supercoiling is presumed to be a mechanism of transcriptional control; however, verification of this hypothesis has been difficult.

Nitrate

Anaerobically, nitrate supplementation induces the nitrate-formate respiratory system and represses other respiratory and fermentative pathways of lower redox potential. The *narGHJI* (nitrate reductase; ref. 36, 62, 137, 216) and *fdhGHI* (FDH-N; ref. 19) operons, both required for nitrate respiration, are induced. Whereas, the *frdABCD* operon (fumarate reductase; ref. 104, 115), the *dmsABC* operon (DMSO and TMAO reductase; ref. 51, 104), the *pfl* gene (190), the *fdhF* gene and the *hyc* operon (25, 171) are repressed in the presence of the high redox potential acceptor, nitrate.

Current evidence suggests that a two-component regulatory system mediates transcriptional control of the *narGHJI*, *fdhGHI*, *frdABCD*, and *pfl* operons. The NAR-X protein (and possibly NAR-Q) displays sequence homology to membrane-bound histidine-protein-kinases (sensors) and NAR-L

to other known DNA-binding proteins (receivers; ref. 81, 164, 220). The NAR-Q protein was proposed by Egan and Stewart (60) as a second protein kinase which could also activate NAR-L via phosphorylation at aspartate residue 59; however, this role has not been confirmed. Mutations in the *narX* gene confer variable phenotypes. In-frame deletions are phenotypically indistinguishable from the wild type (59), while *narX::Tn5* and point mutants relieve (to varying levels) nitrate inhibition at the *frd* operon (60, 118). Kalman and Gunsalus (118) have sequenced three such point mutants which no longer require nitrate for repression and have localized these single-amino acid changes to an 11-residue domain. Molybdate is required for *frd* operon repression in one out of the three *narX*^{*} strains tested, suggesting that NAR-X may have an additional role in molybdenum sensing. The *narL* gene function is less ambiguous, since all mutants studied to date render *frd* operon expression nitrate insensitive (104, 117).

When cells are grown in buffered, rich medium with glucose, *pfl* operon expression is repressed approximately 2.5-fold in the presence of nitrate. The repression is directly mediated by NAR-L and not relieved by the addition of formate (190). The physiological significance of this partial NAR-L repression is unclear since formate is the substrate for nitrate respiration. The NAR-L protein may serve as a modulator of formate levels at the site of *pfl* transcription to prevent formic acid accumulation during nitrate respiration. Transcription of the *hyc* (*hyd-17*) and *fdhF* genes are repressed in the presence of nitrate (171);

therefore, FHL activity is also reduced. However, this control does not appear to be mediated by the NAR-X, NAR-L cascade (218). High concentrations of formate relieve both nitrate and fumarate repression of the FHL structural genes (171). Interestingly, mutations in *narL*, *nark* (hypothetical nitrate transport gene) and *narGHJI* partially relieved nitrate inhibition at the level of *hyc* and *fdhF* operon expression when tested in the absence of formate (218). Likewise, strain WL24, deficient in the FDH-N selenopeptide encoded by the *fdhGHI* operon, is derepressed for the synthesis of FDH-H when grown anaerobically in the presence of nitrate (192). These results suggest that the nitrate effect at the *fdhF* and *hyc* level may be a consequence of formate (an obligate inducer) being channeled to nitrate respiration.

Formate

Irrespective of whether high redox potential electron acceptors (dioxxygen or nitrate) are present in the environment, glucose is actively transported into the cytoplasm by the phosphotransferase system (PTS) and catabolized to pyruvate (for review, see 75). Therefore, the pivotal metabolic step signalling anaerobiosis could potentially be pyruvate degradation by PFL to formate which occurs in the absence of dioxxygen (42). Pyruvate dehydrogenase (PDH) and pyruvate formatelyase (PFL) are tightly controlled by dioxxygen both transcriptionally and allosterically (123, 124, 205). Anaerobiosis represses and inhibits PDH while inducing PFL synthesis and catalytic activity (83, 170). Pyruvate formatelyase constitutes up to 3% of the cytoplasmic protein in

anaerobically growing cells (122). Lactate dehydrogenase (LDH), responsible for the oxidation of pyruvate to lactic acid, is presumed to be only fully active at high concentrations of pyruvate (17).

The pathway of formate oxidation is determined by the presence or absence of nitrate. Anaerobically, in the absence of nitrate, both formate and low pH enhance FHL activity (17, 46, 78). Additionally, formate and dihydrogen are known inducers of hydrogenase synthesis (128, 135, 171). Formate is considered an obligate inducer for expression of the *fdhF* and *hyc* (*hyd-17*) operons. The DNA sequence of both operons has been determined (27, 145, 243), and the structural genes appear to have comparable regulatory patterns in various media and genetic backgrounds tested, as measured by the amount of β -galactosidase activity produced by the appropriate *lac* fusion mutants (25, 171). DNA sequence analysis suggests a common upstream regulatory sequence (URS) positioned between bases -101/-142 (*fdhF*) and -53/-79 (*hyc*) relative to the transcription start site. The URS exhibits several characteristics comparable to eukaryotic enhancer elements (121). The *fdhF* URS consists of two tandem conserved hexanucleotide sequences (GTCACG; ref. 22). Deletion analysis of the *fdhF* upstream region show that the URS is a *cis*-acting DNA element essential for formate induction and dioxygen/nitrate repression (22). Construction of chimeric promoter regions, exchanging the *Klebsiella pneumoniae nif* upstream activating sequence (UAS for nitrogen fixation genes) with the *fdhF* URS and vice versa, established that the *cis*-acting element mediated complete regulatory control of the *fdhF* gene

and not the spacer region (23). The *hyc* operon, on the other hand, is divergently transcribed with the *hyp* operon. The 210 bp intragenic region contains 3 central hexanucleotide repeats (GTCGAC) which are comparable to the *fdhF* URS (145).

Further analysis of both promoters revealed sequence homology to the "-24 and -12" promoter consensus (TGGCAC-N5-TTGC) which is recognized by the σ^{54} subunit of RNA polymerase (24, 227). This subunit is encoded by the constitutive *rpoN* (*ntrA* or *glnF*) gene and is required for expression of a number of operons whose physiological roles are apparently diverse although many are involved in nitrogen metabolism (for review, see 131). The *rpoN* gene product was originally identified as a *trans*-acting factor required for glutamine synthetase transcription in enteric bacteria and is now known to encode a unique sigma factor (69, 132), distinct both structurally and functionally from the major σ^{70} subunit of RNA polymerase. Based on DNA sequence analysis, no significant amino acid homology between the σ^{54} subunit and the major family of sigma subunits was observed (for review, see 89). However, a helix-turn-helix motif can be predicted in the C-terminal end of both sigma factors (152). Mutagenesis coupled with DNA footprinting has shown that the second helix is probably involved in promoter recognition (48, 70, 204). All σ^{54} -dependent promoters, which have been analyzed, are transcriptionally controlled by activators (homologous to the receiver proteins of the two-component systems) which commonly bind to enhancer-like upstream elements. The NTR-C and NIF-A proteins,

transcriptional activators which have been most thoroughly investigated, are essential for catalysis of isomerization from closed to open promoter complexes. This would suggest that the *fdhF* and *hyc* operons require a common *trans*-acting, DNA-binding protein for transcription.

Sankar *et al.* (186) reported the presence of a putative regulatory element encoded by the *fhlA* gene which was required for transcription at both promoters. This was subsequently verified by Schlensog *et al.* (196). The *fhlA* mutant isolated was phenotypically deficient in Fhl activity (FDH-H and HYD-3) which would be expected for a positive regulator of the FHL modulon. Transduction and plasmid complementation analysis revealed that *fhlA* was linked to the *hydEFB* gene cluster (*hyp* operon) and adjacent to the *hydB* gene. "Maxicell" experiments established the apparent molecular weight of FHL-A protein as 78 kDa and suggested transcription occurred both aerobically and anaerobically (186). This present study includes DNA sequence analysis of the *fhlA* gene and predicted amino acid sequence of FHL-A, now known to be homologous to DNA-binding proteins found in two-component regulatory systems. By studying regulation of the *fhlB* operon, it was shown that both formate and FHL-A were necessary for its transcription. Hypothetically, formate could activate the FHL-A protein or an unidentified protein which positively interacts with FHL-A during transcription.

Molybdenum

Five redox enzymes of *E. coli* are known to contain molybdenum (Mo) as MPT: FDH-H, FDH-N, nitrate reductase, TMAO/DMSO reductase, and

biotin-sulfoxide reductase (55, 92, 111, 140, 141, 202, 219; for review, see 217). Molybdopterin is a complex composed of a nonprotein organic moiety (6-alkyl-pterin) with a Mo atom (111, 113). Initially, Pateman *et al.* (169) linked anaerobic chlorate-resistance to nitrate respiration in *Aspergillus nidulans* by demonstrating that the nitrate-respiration defective mutant is unable to reduce chlorate to toxic chlorite. The chlorate-resistance (*chl*) genes exhibited pleiotropic phenotypes and were later identified as essential for molybdenum transport, regulation and formation of functional molybdopterin containing enzymes (for review, see 94). Complementation analysis and deduced sequence homology to other periplasmic binding and transport systems estimated at least 11 *chl* genes mapping at 5 loci which are necessary for MPT biosynthesis in *E. coli* (110, 178).

The *chlA* operon, consisting of three complementation groups (18 min), and the *chlE* operon, composed of two (18 min), are presumed to be necessary for synthesis of the organic portion of the MPT (11, 113, 178, 219). Mutations in either one of these operons give rise to defects in pterin biosynthesis (114). Baker and Boxer (14) constructed merodiploids containing *chlA*⁺ / Φ (*chlA*'-'*lacZ*⁺) to study the transcription of the *chlA* locus. Various *chl* mutations (*chlA*, *chlB*, *chlD*, *chlG* and *chlE*) were introduced into the merodiploid strains. These results suggested that the *chlA* operon is anaerobically inducible and repressed in the presence of MPT. These investigators concluded

that *chlA* repression is probably mediated by a complex of MPT and a MPT-binding protein which has been reported previously (6, 189).

The constitutively synthesized *chlB* gene (86 min) product has been purified ($M_r = 35$ kDa) and is denoted as "association factor- F_A " which is essential for synthesis of functional MPT (143, 180). A mutation in this gene inhibits insertion of the MPT into the apoprotein; therefore, association factor- F_A may have a direct role in mediating the terminal step of apoprotein maturation (71, 146). Johnson *et al.* (112) recently developed a more sensitive fluorescence technique to analyze cell extracts for MPT and MGD derivatives. By comparing *chlB* mutant extracts to wild type, it was concluded the *chlB* gene was required for the formation of MGD, a step occurring late in the MPT maturation process. The *chlC* (27 min) and *chlF* (32 min) operons code for nitrate reductase and FDH-N respectively. Since neither are necessary for MPT biosynthesis, they have been renamed *narGHJI* (*chlC*) and *fdnGHI* (*chlF*) (11, 19, 219). Mutations in both the *chlG* (0 min) and *chlD* operons (17 min) are suppressed by Mo supplementation of the growth medium (11, 207, 219). Phenotypically, the *chlG* mutants lack nitrate reductase activity, even though detectable levels of the protein were produced in the original isolates, and retain wild type levels of Fhl activity (219). It is currently postulated that the CHL-G protein functions to incorporate Mo into molybdoenzymes and Mo-binding proteins (94).

Mutations in the *chlD* operon can be phenotypically distinguished from the *chlG* gene. Dihydrogen production is lacking in *chlD* mutants

due to a deficiency in both FDH-H and HYD-3 activities (33, 73, 76, 196). The partial sequence of the *chLD* operon discloses significant homology to binding-protein-dependent transport systems (110). Generally, this type of transport is a unidirectional process. It is now known that this process requires ATP for energy instead of acetyl-phosphate (20, 95, 97, 100), and the substrate does not undergo modification during transport (4). In the systems which have been analyzed, the number and location of proteins involved in the transport process are conserved (for review, see 5, 52). These proteins include: (i) a high-affinity, substrate-binding protein located in the periplasmic space which is released upon cold osmotic shock (i.e. HIS-J, MAL-E), (ii) two integral membrane proteins present in lower concentration than the binding protein (i.e. HIS-Q, HIS-M, MAL-F, MAL-G), and (iii) another membrane-associated protein, presumed to be peripheral, which contains a nucleotide-binding domain with a conserved amino acid sequence (i.e. HIS-P, MAL-K). The central domain of the complete CHL-D sequence revealed extensive homology to peripheral transport proteins (110). The partial CHL-J sequence suggested that this protein is an integral membrane component of Mo transport (110).

Both the *chLD* gene and the *molR* gene, recently described by Lee *et al.* (136) are essential for *fdhF* and *hyc* (*ant*, *hyd-17*) operon expression as measured by β -galactosidase activity (134, 196). This implies that transcription of these FHL operons either directly or indirectly requires Mo or a Mo-derivative. Interestingly, both *molR* and *chLD* genes

are located in the same operon as determined by transduction and plasmid complementation analysis (Shanmugam, unpublished results; this study). However, MOL-R is expressed constitutively (134) while CHL-D synthesis is Mo repressible (155) thus suggesting at least two promoters for this operon.

Nickel

E. coli synthesizes three distinct nickel (Ni) containing hydrogenase isoenzymes (12, 193). The HYD-1 protein and an enzymatically active fragment of HYD-2 have been purified to homogeneity (1, 13, 66, 168, 194). Biochemical characterization of these proteins revealed that HYD-1 and HYD-2 contain nonheme iron, inorganic sulfur and nickel. The HYD-3 protein, responsible for dihydrogen evolution, is electrophoretically labile and has remained elusive to purification (193). DNA sequence analysis of the large subunit of HYD-3 (*hyc* ORF5), however, displays significant homology to the "nickel-amino acid" consensus sequence of other NiFe-hydrogenases (27, 195, 214). The large subunits of all sequenced NiFe-hydrogenases contain the consensus R-X-C-G-X-C-X₃-H in the amino-terminus and D-P-C-X₂-C-X₂-H at the carboxy-terminus (177). Apparently the conserved sites at both termini are somehow involved in liganding nickel at the active site (61, 87).

Several genes required for total hydrogenase activity (thus Fhl activity) have been characterized and are presumed to be involved in nickel transport, nickel processing, or hydrogenase regulation. These include *hydA* (59 min), *hydB* or *hypE* (59 min), *hydC* (77.6 min), *hydD*

(77.6 min), *hydE* or *hypB* (59 min), and *hydF* or *hypD* (59 min; ref. 35, 119, 128, 135, 185, 187, 188, 231, 238). Although the *hyp* operon has been sequenced, the DNA did not display any detectable homology to the DNA available in Genbank data base and thus possible function could not be deduced (144). Mutations in both the *hydC* and *hydE* genes are suppressed by high concentrations of nickel in the growth medium (231, 238). This would suggest that both are essential for transport of the divalent metal ion. Transcription of the FNR-controlled *hydC* is fully repressed by approximately 0.2 mM NiCl_2 which is correlated to the Ni-dependent restoration of hydrogenase activity (239). Hypothetically, a nickel-protein complex could function as a transcriptional repressor at the *hydC* gene operator. The *hydE* or *hypB* gene, now known to be a component of the *hyp* operon, does not appear to be nickel repressible at either of its two promoters (FNR or σ^{54} -dependent), as measured by RNA transcript levels and β -galactosidase activity of *hyp* gene fusions (144, this study). It is now believed that this gene product is required for activation or processing of nickel. The other *hyd* genes may also process the nickel for insertion into hydrogenase or regulate the expression of all three isoenzymes. Sankar *et al.* (187, 188), using maxicells, determined that both *hydB* (*hypE*) and *hydF* (*hypD*) expression was *rpoN* dependent, anaerobically inducible, and nitrate repressible. Fumarate and TMAO supplementation had no significant effect on transcription of either gene. This is in correlation with *hyp-lac* gene

fusion expression and transcript levels under the same conditions (144; this study).

Recent studies have established that nickel is not essential for transcription of the electrophoretically stable HYD-1 and HYD-2 isoenzymes, but necessary for hydrogenase activity. Lutz *et al.* (144) reported that mutations in *hypBCDE* did not alter hydrogenase transcription as monitored by immunoblotting analysis with anti-HYD-1 and HYD-2 antibodies; however, the electrophoretic mobility was altered in a 10% SDS-polyacrylamide gel. Potentially the lack of nickel processing and insertion could lead to a modified hydrogenase conformation and proteolytic susceptibility. Menon *et al.* (151) obtained similar results for HYD-1 in a *hydE* (*hypB*) mutant. Although HYD-1 transcription was comparable to wild type in these mutants in the absence of Ni, activity was absent. Additionally, the *hydE* gene product appeared essential for membrane localization of HYD-1, and this requirement was nickel repressible. Labile HYD-3 is probably regulated by a similar post-translational mechanism requiring nickel incorporation for Fhl activity.

Selenium

A number of enzymes from both prokaryotic and eukaryotic organisms, including FDH-H, require selenium in the form of selenocysteine for activity (172; for review, see 26). DNA sequence analysis of the *fdhF* gene [coding for the 701 amino acid FDH-H subunit ($M_r=79$ kDa)] established that a UGA "nonsense" codon at amino acid

position 140 with an associated unique stem loop structure is essential for selenocysteinyl-tRNA incorporation during translation (15, 64, 65, 243, 244). By constructing a plasmid in which the first 39 amino acids of FDH-H were fused to β -galactosidase, it was determined that selenium was not required for anaerobic transcription of the *fdhF* gene (243). Selenium regulation of Fhl activity is evidently at the translational level of the *fdhF* gene.

In summary, the FHL system is presumed to be regulated by a number of elements including anaerobiosis, nitrate, formate, low pH, molybdate, nickel, and selenium. The absence of dioxygen induces the transcription of *fdhF*, *hyc*, and other operons whose products are required for FHL activity. Additionally, enzymes necessary for dihydrogen production are often irreversibly inactivated by the presence of dioxygen thus enabling tighter metabolic control. Nitrate repression of FHL appears to be mediated by the NAR-X and NAR-L, two-component regulatory system at the level of *pfl* gene transcription. Therefore, it is possible that the nitrate repressive effect on the transcription of FHL structural components is indirectly a result of the absence of the obligate inducer, formate as well as low pH. Currently it is unknown whether molybdenum is directly or indirectly required for Fhl transcription. Mutations in both *molR* and *chlD* genes abolish expression of *fdhF* and *hyc* operons and are reversed by high molybdate. Nickel is essential for HYD-3 activity at the post-translational level; whereas, selenium is required for translation of the selenopolypeptide, FDH-H.

In this study, the mechanism by which formate and molybdenum regulate the FHL pathway was investigated. This includes isolation of a specific mutant, strain SE-2011 [$\Phi(fhlB\text{'-'}lacZ^+)$], physiological characterization of this *fhlB* mutant, and *trans*-acting factors regulating the *fhlB* gene. These *trans*-acting factors were found to be the FHL-A protein of the formate-dependent pathway and an uncharacterized component of the molybdate-dependent pathway.

MATERIALS AND METHODS

Materials

Biochemicals were purchased from Sigma Chemical Co. Analytical-grade inorganic and organic chemicals were from Fisher Scientific.

Bacterial Strains and Media

The bacterial strains are derivatives of *E. coli* K-12 and are listed in Table 1. Basal minimal, dihydrogen/fumarate and glycerol/fumarate media and LB were prepared as described previously (135). Cultures were grown at 37°C in LB which was supplemented with glucose (0.3%) or sodium formate (0.1% to 0.5%), as needed. Ampicillin (100 µg/ml), kanamycin (50 µg/ml), streptomycin (100 µg/ml), tetracycline (15 µg/ml), chloramphenicol (5 µg/ml) or X-gal (20-40 µg/ml) were added as needed.

Isolation of Mutants

Strain MC4100, grown in LB+maltose (0.3%; LBM) medium was mutagenized with λ lacMu53 and λ pMu507, as described by Bremer *et al.* (29). Kanamycin resistant mutants were transferred by replica plating techniques to LB + X-Gal medium (156) and incubated under aerobic or anaerobic conditions. Mutants which are Lac⁺ only under anaerobic growth conditions were identified and inoculated into 1 ml of LBG medium in 12 x 75 mm tubes. These tubes were sealed with serum stoppers and

Table 1. Bacterial strains used in this study

Strain	Relevant Genotype or Phenotype	Source or Reference
<i>E. coli</i>		
BW545	$\Delta(lacU)169$, <i>rpsL</i>	G. Walker (242)
CSH26	<i>ara</i> , $\Delta(lac-pro)$, <i>thi</i>	Laboratory collection
JRG780	<i>trpA9761</i> , <i>frdA11</i> , <i>trpR72</i> , <i>gal-25</i> , <i>rpsL195</i>	CGSC #5916
JRG861a	<i>gal</i> , <i>trpA9761</i> , <i>iclR</i> , <i>trpR</i> , <i>rpsL</i> , <i>fnr</i>	J. Guest
LCB898	<i>thr-1</i> , <i>leuB6</i> , <i>pfl-1</i> , <i>thi-1</i> , <i>lacY1</i> , <i>rpsL175</i> , <i>tonA21</i>	CGSC #6161
LS853	<i>trpA9605</i> , <i>his-85</i> , <i>cya-2</i> , <i>trpR55</i>	CGSC #5381
M2508	Hfr, <i>relA1</i> , <i>spoT1</i> , <i>metB1</i> , <i>mela7</i>	CGSC #4926
M9s	MC4100, $\Phi(fdhF'-lacZ^+)$	A. Böck (171)
MC4100	<i>araD139</i> , $\Delta(argF-lacU)205$, <i>ptsF25</i> , <i>relA1</i> , <i>rpsL150</i> , <i>deoC1</i> , <i>flb5301</i>	CGSC #6152
MJ-2	$\Phi(fhlB'-lacZ^+)$, <i>hydF102</i> , <i>cys::Tn10</i>	P1 transduction (SE-2011 x SE-67-1)
MJ-3	MC4100, $\Phi(fhlB'-lacZ^+)$	P1 transduction (MC4100 x SE-2011)
MJ-4	BW545, $\Phi(fhlB'-lacZ^+)$	P1 transduction (BW545 x SE-2011)
MJ-5	$\Phi(fhlB'-lacZ^+)$, <i>rpoN::Tn10</i>	P1 transduction (SE-2011 x YMC18)
MJ-6	$\Phi(fhlB'-lacZ^+)$, <i>fnr</i> , <i>zcyj-5::Tn10</i>	P1 transduction (SE-2011 x SE-1188)
MJ-7	$\Phi(fhlB'-lacZ^+)$, <i>narL215::Tn10</i>	P1 transduction (MJ-4 x RK5278)

Table 1. continued

Strain	Relevant Genotype or Phenotype	Source or Reference
MJ-8	$\Phi(fhlB'-lacZ^+)$, <i>cya-2</i> , <i>zif-4::Tn10</i>	P1 transduction (SE-2011 x SE-1162)
MJ-9	$\Phi(fhlB'-lacZ^+)$, <i>pfl-1</i> , <i>zba-6::Tn10</i>	P1 transduction (SE-2011 x SE-1265)
MJ-18	MJ-19, Hfr PO(<i>fhlB</i>)	Conjugation (MJ-19 x TT627)
MJ-19	CSH26, $\Phi(fhlB'-lacZ^+)$	P1 transduction (CSH26 x SE-2011)
MJ-20	$\Phi(fhlB'-lacZ^+)$, <i>fhlA::Tn10</i>	P1 transduction (SE-2011 x SE-1174)
MJ-21	SE-1000, <i>metB1</i> , <i>melA7</i> , <i>thr</i> ⁺ , <i>arg</i> ⁺ , <i>leu</i> ⁺ , F ⁻	Conjugation (SE-1000 x M2508)
MJ-40	$\Phi(fhlB'-lacZ^+)$, <i>molR</i>	P1 transduction (SE-2011 x SE-1704)
MJ-50	SE-1000, $\Phi(fhlB'-lacZ^+)$	P1 transduction (SE-1000 x SE-2011)
MJ-101	$\Phi(fhlA'-lacZ^+)$, <i>rpoN::Tn10</i>	P1 transduction (SE-2007 x YMC18)
MJ-102	$\Phi(fhlA'-lacZ^+)$, <i>narL215::Tn10</i>	P1 transduction (SE-2007 x RK5278)
MJ-103	$\Phi(fhlA'-lacZ^+)$, <i>fnr</i> , <i>zcj-5::Tn10</i>	P1 transduction (SE-2007 x SE-1188)
MJ-107	$\Phi(fhlA'-lacZ^+)$, <i>cya-2</i> , <i>zif-4::Tn10</i>	P1 transduction (SE-2007 x SE-1162)
MJ-108	$\Phi(fhlA'-lacZ^+)$, <i>molR</i>	P1 transduction (SE-2007 x SE-1704)
MJ-109	$\Phi(fhlA'-lacZ^+)$, <i>pfl-1</i> , <i>zba-6::Tn10</i>	P1 transduction (SE-2007 x SE-1265)

Table 1. continued

Strain	Relevant Genotype or Phenotype	Source or Reference
PC0287	<i>thr-20, leu-32, proA35, argF58, argI60, lacY1, gal-6, rpsL125, tonA48, tsx-70, supE44</i>	CGSC #5404
RK5278	<i>narL215::Tn10</i>	V. Stewart
SE-1000	<i>cysC-43, srl-3000::Tn10, thr-1, leu-6, thi-1, lacY-1, galK2, ara-14, xyl-5, mtl-1, proA2, his-4, argE3, rpsL31, tsx-33, supE44</i>	Laboratory collection
SE-67-1	<i>hydF102, cys::Tn10</i>	P1 transduction (SE-67 x SE-1300)
SE-1100	BW545, $\Phi(\text{molR}^+ \text{'-'} \text{lacZ}^+)$	Laboratory collection (136)
SE-1162	LS853, <i>zif-4::Tn10</i>	Laboratory collection
SE-1174	<i>fhlA::Tn10</i>	Laboratory collection (186)
SE-1188	JRG861a, <i>zcg-5::Tn10</i>	Laboratory collection
SE-1265	LCB898, <i>zba-6::Tn10</i>	Laboratory collection
SE-1300	BW545, <i>cys::Tn10</i>	Laboratory collection
SE-1651	$\Phi(\text{hyp}^+ \text{'-'} \text{lacZ}^+)1, fnr, zcg-5::Tn10$	P1 transduction (SE-2001 x SE-1188)
SE-1652	$\Phi(\text{hyp}^+ \text{'-'} \text{lacZ}^+)2, fnr, zcg-5::Tn10$	P1 transduction (SE-2002 x SE-1188)
SE-1654	$\Phi(\text{hyp}^+ \text{'-'} \text{lacZ}^+)1, narL215::Tn10$	P1 transduction (SE-2001 x RK5278)

Table 1. continued

Strain	Relevant Genotype or Phenotype	Source or Reference
SE-1655	$\Phi(hyp^{-}-'lacZ^{+})_2$, <i>narL215::Tn10</i>	P1 transduction (SE-2002 x RK5278)
SE-1657	$\Phi(hyp^{-}-'lacZ^{+})_1$, <i>rpoN::Tn10</i>	P1 transduction (SE-2001 x YMC18)
SE-1658	$\Phi(hyp^{-}-'lacZ^{+})_1$, <i>rpoN::Tn10</i>	P1 transduction (SE-2001 x YMC18)
SE-1659	$\Phi(hyp^{-}-'lacZ^{+})_2$, <i>rpoN::Tn10</i>	P1 transduction (SE-2002 x YMC18)
SE-1660	$\Phi(hyp^{-}-'lacZ^{+})_2$, <i>rpoN::Tn10</i>	P1 transduction (SE-2002 x YMC18)
SE-1704	<i>molR::Tn5</i> , <i>zgg::Tn10</i>	Laboratory collection
SE-1714	$\Phi(fhlB^{-}-'lacZ^{+})$, <i>chlD::Tn10</i>	P1 transduction (SE-2011 x VJS720)
SE-1760	$\Phi(hyp^{-}-'lacZ^{+})_1$, <i>chlD::Tn10</i>	P1 transduction (SE-2001 x VJS720)
SE-1761	$\Phi(hyp^{-}-'lacZ^{+})_2$, <i>chlD::Tn10</i>	P1 transduction (SE-2002 x VJS720)
SE-1762	$\Phi(fhlA^{-}-'lacZ^{+})$, <i>chlD</i>	P1 transduction (SE-2007 x VJS720)
SE-2001	MC4100, $\Phi(hyp^{-}-'lacZ^{+})_1$	This study
SE-2002	MC4100, $\Phi(hyp^{-}-'lacZ^{+})_2$	This study
SE-2007	MC4100, $\Phi(fhlA^{-}-'lacZ^{+})$	This study
SE-2009	MC4100, $\Phi(hydC^{-}-'lacZ^{+})$	This study
SE-2011	MC4100, $\Phi(fhlB^{-}-'lacZ^{+})$	This study
VJS720	<i>chlD::Tn10</i>	V. Stewart

Table 1. continued

Strain	Relevant Genotype or Phenotype	Source or Reference
YMC18	<i>endA</i> , <i>thi</i> , <i>hsdR</i> , $\Delta(lacU)169$, <i>rpoN</i> :Tn10	B. Magasanik
<i>Salmonella typhimurium</i>		
TT627	<i>strA1</i> , <i>pyrC7</i> /F'ts114 <i>zzf</i> :Tn10	J. Roth (40)

the gas phase was replaced with dinitrogen. After 16 hr of incubation at 37°C, dihydrogen in the gas phase of the culture tubes was determined using a gas chromatograph (Varian; Model 920) fitted with a 50 nm molecular sieve column. From a total of 68 mutants, 13 were found to be defective in dihydrogen production (Fhl^-) and were analyzed further.

Enzyme Activities and their Respective Culture Conditions

β -galactosidase activities and culture conditions. For anaerobic induction of β -galactosidase activity in mutant strains SE-2001, SE-2002 and SE-2011, 120 ml of medium in a 160 ml "Wheaton" bottle was inoculated (1% V/V) with a 1.5 hr old aerobic culture, grown at 37°C, in a shaker, at 250 rpm. However, with strain SE-2007 or derivatives thereof, alternate procedures were used to maximally aerate the culture at low cell density before starting the experiment. A 2 hr old aerobic culture (37°C; 250 rpm) was transferred to fresh LB medium (1% V/V) and grown again in the shaker for 1 hr. This culture was used to inoculate the experimental medium at 10% (V/V) and then grown under anaerobic conditions in a "Wheaton" bottle. The bottles were closed with rubber stoppers and secured with aluminum seals. The gas phase was replaced with argon. Samples were removed at different time periods with a syringe and needle and growth of the culture and β -galactosidase activity of the cells were determined. In another set of experiments, the aerobic cultures were used to inoculate (1% V/V) the appropriate medium in 13 x 100 mm screw cap tubes filled to the top. Cells from

these cultures were harvested after 4 hr of incubation at 37°C (standing) and used for enzyme assays.

The amount of β -galactosidase present in cells was determined as described by Miller (156), after permeabilization with SDS and chloroform. The specific activity of the enzyme is expressed as nanomoles ortho-nitrophenol produced per min per mg cell protein. The differential rate of synthesis of β -galactosidase activity was calculated as units of activity per μ g cell protein produced by the culture and represents the increase in the amount of β -galactosidase activity produced by the culture in relation to the increase in total cell protein.

Dihydrogen related metabolic activities and culture conditions.

Whole cells were utilized for all biochemical determinations (135). Cells were inoculated (5% V/V) from overnight LB-grown cultures into fresh LBG medium in 250 ml screw cap bottles or 20 ml screw cap tubes (16 x 150 mm) filled to the top. Cultures were grown anaerobically for 4 hr at 37°C. Cells were collected by centrifugation (3,000 x g for 10 min) at 4°C, washed with half volume of phosphate buffer (0.1 M sodium phosphate, pH 7.0; 1 mM glutathione; 0.1 mg/ml chloramphenicol). The cell pellet was resuspended in 1.0 ml buffer and diluted to 1.75 mg cell protein per ml, as determined in a Spectronic 710 spectrophotometer (Baush and Lomb) in which 1 A_{420nm} unit equalled 350 μ g cell protein per ml. Samples were maintained under N_2 at 4°C and assayed immediately.

Total hydrogenase activity, measured as tritium exchange (7, 138), was determined using 50 μ g cell protein adjusted to 0.2 ml volume in sodium phosphate buffer (0.1M; pH 7.0). Assays were carried out in 12 x 75 mm thick walled test tubes, sealed with 11 x 17 mm serum stoppers. The gas phase was replaced with helium. The reactions were initiated with 0.7 ml of dihydrogen and 0.55 μ Ci tritium gas (11.2 mCi/mmol; New England Nuclear Corp.) and terminated after 1 hr incubation at room temperature. After removing the stopper, the reaction mixture was agitated with a vortex mixer and allowed to stand for 10 min. Tritium in a 50 μ l sample in 2.5 ml Scintiverse-E scintillation fluid was determined, and total hydrogenase activity was calculated as nanomoles of tritiated water produced per min per mg cell protein.

Hydrogen uptake (HUP) was measured both as the ability to reduce benzyl viologen (BV) (96, 167) and fumarate with dihydrogen as electron donor (148). The reduction of BV was monitored in 13 x 100 mm test tubes with "subaseal" stoppers. The assay mixture (0.1 M sodium phosphate buffer, pH 7.0; 4 mM BV) was degassed, the gas phase replaced with dihydrogen and reduced with sodium dithionite until a faint purple color appeared. The reaction was initiated by the addition of whole cells to a final volume of 2.5 ml. The rate of BV reduction was correlated to ΔA_{550nm} using a DW2C spectrophotometer (SLM Instruments; Urbana, Illinois). The HUP_{BV} activity was expressed as nanomoles BV reduced per min per mg cell protein. Fumarate reduction was measured as the rate of dihydrogen consumption from the gas phase using a gas

chromatograph as described above. Assay mixture consisted of 0.1 M phosphate buffer (pH 7.0) and 50 mM fumarate brought to a final volume of 1.0 ml with cells. "Wheaton" vials (10 ml) with serum stoppers and aluminum seals were used to establish anaerobic conditions (10% H₂/90% N₂ gas phase). The HUP_{fumarate} activity was calculated as nanomoles of H₂ consumed per min per mg cell protein.

Formate-dependent reduction of BV (FDH-H, FDH activity associated with FHL), was assayed in an assay mixture containing 0.33 M sodium phosphate buffer (pH 7.0), 6.5 mM BV, 40 mM formate and cells in a final volume of 4.0 ml (135). Test tubes (13 x 100 mm) with "subaseal" stoppers were used to monitor the reaction in a N₂ atmosphere. Assay mixture was reduced with sodium dithionite until a faint purple color developed. Reaction was initiated by adding whole cells and the ΔA_{550nm} was correlated to BV reduction. The FDH-H activity was calculated as nanomoles BV reduced per min per mg of cell protein.

Formate hydrogenlyase activity was measured in 70 mM sodium phosphate buffer (pH 6.5) containing 0.1 M formate (final volume of 1.0 ml with cells; 96). "Wheaton" vials (10 ml) with serum stoppers and aluminum seals were used to monitor the production of dihydrogen from formate in a dinitrogen atmosphere. Dihydrogen evolution was quantified using a gas chromatograph. The FHL activity was calculated as nanomoles of H₂ produced per min per mg of cell protein.

Fumarate reductase activity was assayed in 92.5 mM sodium phosphate buffer (pH 7.0), 30 mM fumarate, and 0.35 mM BV under a N₂

atmosphere. Activity was measured as fumarate-dependent oxidation of BV_{red} . Assay mixture was reduced with sodium dithionite to 2.0 absorbance units at 550 nm, whole cells were added to a final volume of 5.0 ml, and oxidation of reduced BV was monitored as ΔA_{550nm} . Activity was expressed as nanomoles BV_{red} oxidized per min per mg cell protein (208).

Chloramphenicol acetyltransferase (CAT) assay and culture conditions. Plasmid pSV208 (*fdhF'*::*'cat*) was generously provided by Dr. A. Böck (196). Cell extract was prepared according to Brosius and Lupski (30) with modifications. Transformants were inoculated into 1.0 ml LB + ampicillin (50 μ g/ml) medium and grown to stationary phase. This was used as an inoculum for 20 ml (5% V/V) of the same medium supplemented with glucose (0.3%), formate (0.2%), and trace metals ($FeSO_4 \cdot 7H_2O$, 0.01 mg/ml; $NaMoO_4 \cdot 2H_2O$, 0.01 mg/ml; $NaSeO_3 \cdot 5H_2O$, 0.263 ng/ml). The cultures were grown anaerobically (16 x 150 mm screw cap tubes filled to top) to approximately 5×10^8 CFU/ml (4 hr; 37°C). Cells were harvested by centrifugation at 5,000 rpm for 10 min at 4°C, washed with 20 ml of assay buffer (50 mM Tris-HCl, pH 7.8; 30 μ M dithiothreitol), and resuspended in 1.0 ml of same buffer. This was transferred to 1.5 ml plastic centrifuge tubes and placed at -70°C for 1 hr. Cells were thawed at 37°C and then disrupted by sonication (one 20 seconds pulse at full power; Heat Systems sonifier with microprobe) after dilution to 2.0 ml volume in conical-bottomed glass test tubes in an ice-water bath. Cellular debris were removed by centrifuging in

"Eppendorf" micro-centrifuge for 5 min at 4°C. Supernatant was collected and kept on ice for immediate assay using a modified procedure described by Shaw (200). The reaction mixture was freshly prepared by dissolving 4 mg of 5,5' dithiobis-2-nitrobenzoic acid (DTNB) in 1.0 ml Tris-HCl (pH 7.8), adding 0.2 ml of 5 mM acetyl-CoA, and then making the total volume up to 10 ml. After measuring the rate of change of absorbance at 412 nm with 900 μ l of reaction mixture and 80 μ l of extract, the reaction was started with 20 μ l of chloramphenicol (Cm; 5 mM in 70% ethanol) added to a final concentration of 0.1 mM. The difference in the rate of change at $A_{412\text{nm}}$ with and without Cm was calculated. Protein concentration of the extract was determined using Coomassie brilliant blue (28). The CAT activity was expressed as nanomoles of free 5-thio-2-nitrobenzoate produced per min per mg cell protein.

Genetic and Molecular Biological Experiments

Bacteriophage (P1 and lambda) preparation by plate lysis. Host strain was grown to stationary phase in LB medium for P1 infection. Cells were sedimented by centrifugation (3,500 $\times$ g) at 25°C and resuspended in an equal volume of P1 adsorption medium (5 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; 10 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$). Bacteriophage P1 (10^5 to 10^6 PFU) was added to 0.2 ml of host cells and incubated for 5 min at 25°C. Then 3 ml of LCTG soft agar (5g/L yeast extract; 10 g/L each of NaCl and trypticase peptone; 2.5 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; 25 mg/ml thymine; 60 mM glucose; 0.6%

agar) at 50°C was added to the mixture and after mixing was overlayed on LB+thymine (25 µg/ml) plates. After 9 hr of incubation at 42°C, the overlay from the plates with confluent lysis was harvested after addition of 2.5 ml P1-diluent (10 g/L trypticase peptone; 10 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$). Chloroform (0.5 ml) was added to the lysate and mixed with a glass pipet. This was centrifuged (12,100 x g) for 15 min at 4°C. The supernatant was again extracted with chloroform, and the final supernatant was stored at 4°C with 0.1 ml chloroform. Two consecutive series of P1 infection were done to ensure enrichment of host strain mutation.

Procedures implemented for replication of bacteriophage λ were comparable to P1 with some critical exceptions. Strain LE392 (*supF*, *supE*) was used as the host strain and was grown in LBM medium. Cells were pelleted and resuspended in an equal volume of 10 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Water-Thy agar (0.6% agar; 0.1 mg/ml thymine) was used as the soft agar overlay, and plates were incubated at 37°C. Lambda diluent (10 mM Tris-HCl, pH 7.5; 10 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 50 mM NaCl; 0.1% gelatin) was used to titer and harvest phage.

Transduction using bacteriophage P1 Cm *clr100*. Transduction experiments were carried out according to Miller (156) with modifications. Two milliliters of a "mid-log phase" culture of recipient cells were centrifuged (3,500 x g) for 5 min at 25°C and resuspended in 1.0 ml of P1 adsorption medium. Then 0.2 ml of the resuspension was infected at a M.O.I. of 1 to 10 with the appropriate

phage preparation. After 30 min at 25°C, 1 ml of P1 diluent was added and the bacteria-phage mixture was vortexed. This was then centrifuged (3,500 x g) for 5 min at 25°C, resuspended in 0.1 ml of LB and incubated for 1 hr at 25°C before plating on selective medium for transductants (25°C). Transduction of donor mutation was confirmed prior to and after curing recipients off bacteriophage at 42°C.

Conjugation. The F' complementation analysis was performed according to Miller (156) using F'143-1 (57 to 60 min), F'126 (17 to 30 min), F'116 (60 to 65 min), F'128 (6 to 8 min; *lacZ::Tn10*), F'112 (90 to 98 min) and F'104 (0 to 7 min) provided by Dr. B. Bachmann. The Hfr-mediated transfer of chromosomal DNA was accomplished by aerobically (250 rpm) growing the donor and recipient strains to approximately 5×10^8 CFU/ml at 37°C. Mating was initiated by mixing 1.0 ml of donor with 1.0 ml of recipient in 3.0 ml of fresh LBG (125 ml flask; 50 rpm). Immediately upon removal, samples were vortexed, diluted in minimal medium and centrifuged (3,500 x g) for 5 min at 25°C. Exconjugants were then selected on appropriate media.

Transformation by CaCl_2 -heat shock method. Routine transformation was performed by two methods, depending on the amount of competent cells required. Both were a modification of the calcium chloride-heat shock method described by Mandel and Higa (149) which was later established for transforming episomal elements (43). The first method was basic and expedient. An overnight LB culture of the desired host strain was inoculated into fresh medium in 13 x 100 mm tubes (0.02 ml into 2.0 ml)

and incubated for 2 hr standing at 37°C. Cells were sedimented (3,500 x g; 5 min; 25°C) and resuspended in 0.4 ml of 0.1 M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. DNA (about 50 ng) was added to 0.2 ml of cell suspension and incubated on ice for 20 min. This was transferred to 42°C for 2 min and then returned to ice for 10 min. Fresh LB medium (1.0 ml) was added, and cells were incubated at 37°C for 1 to 2 hr and then plated on applicable selection medium. If more cells were needed, the overnight culture was inoculated into 10 ml LB medium and aerobically grown at 30°C for 1.5 to 2 hr. This was transferred to 37°C for 30 min and then harvested (3,500 x g; 5 min; 25°C). After washing with 0.1 M NaCl, the cells were resuspended in equal volume of 0.1 M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and incubated for 20 min at 25°C. This was then pelleted and resuspended in CaCl_2 solution at approximately one-fifth the original volume. Similar procedures were then followed to that described above.

For high efficiency transformation, competent cells were prepared by a modified procedure and stored at -70°C (Sankar, personal communication). An overnight culture was used to inoculate 50 ml of fresh LB medium. This was grown to early-log phase (2×10^8 CFU/ml) aerobically at 37°C. The cells were centrifuged (3,000 x g; 5 min; 4°C), washed in 0.1 M cold $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, resuspended in equal volume of 0.1 M cold $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and incubated on ice 20 min. Treated cells were then pelleted (3,000 x g; 5 min; 4°C) and resuspended in one-tenth the starting volume with 0.1 M CaCl_2 containing 15% glycerol. This was aliquoted and stored at -70°C for later use. Cells were thawed slowly

on ice and 0.1 ml was transferred to a tube for transformation. DNA (approximately 50 ng) was added to the competent cells. This was incubated on ice for 20 min, transferred to 37°C for 5 min, and returned to ice for 2 min. Transformants were preincubated in SOC medium (20 g/L trypticase peptone; 5 g/L bacto-yeast extract; 10 mM NaCl; 2.5 mM KCl; 10 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$; 10 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 20 mM glucose) with aeration at 37°C for 1 hr prior to plating on selection medium.

Transposon Tn5 mutagenesis of cloned genes in plasmid DNA. The two transposon Tn5 derivatives of plasmid pSE-133 were constructed as described before (188). Plasmid pSE-133 which carries the *hydB*⁺ and *fhIA*⁺ genes was described previously (187). Strain MBM7014 (*supF*) was utilized as the host strain for plasmid DNA (pSE-133) Tn5 mutagenesis. A transformant was aerobically grown (250 rpm) in LBM medium (20 ml) to mid-log phase (3×10^8 CFU/ml) at 37°C, the culture was centrifuged (3,500 x g; 25°C), and the pellet was resuspended in 1.0 ml of 10 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Cells were infected with λ NK421 (Tn5) at a M.O.I. of 10 for 30 min at 25°C. The infected cells were then vortexed, centrifuged (3,500 x g; 25°C), washed with 5.0 ml of LB medium, and resuspended in final volume of 10 ml. This was subcultured (2.0 ml inoculum) in 10 ml fresh LBG medium supplemented with 10 mM sodium citrate and aerobically grown (250 rpm) at 37°C for 30 min and then shifted to 30°C for 1 hr. Kanamycin (50 $\mu\text{g/ml}$) and ampicillin (100 $\mu\text{g/ml}$) were added after the hr. Then the culture was grown to stationary phase (18 hr), and plasmid DNA was extracted by the alkaline lysis method described below (150).

Plasmids carrying the transposon Tn5 in the gene of interest were selected as Km^r Amp^r transformants and then screened for lack of complementation in the appropriate mutant. Location of the transposon Tn5 was determined by analyzing "restriction endonuclease" digests of the DNA. The relevant genotype of plasmid pSE-133-1 is *hydB::Tn5*, *fhlA*⁺ and pSE-133-2 carries the transposon in the *fhlA* gene (*hydB*⁺, *fhlA::Tn5*).

Plasmid and chromosomal DNA preparations. Small and large scale plasmid isolation were carried out following standard alkaline lysis protocol with some modifications (150). The cesium chloride gradient consisted of (final concentration) 1 g/ml cesium chloride and 0.625 mg/ml ethidium bromide. This was centrifuged at 50,000 rpm (160,000 x g), for 18 hr, at 18°C. Plasmid band was recovered with syringe fitted with a 22G x 1" needle, and ethidium bromide was removed with water saturated 1-butanol. Extracted plasmid solution was diluted with 2 volumes of deionized water and then ethanol precipitated at -20°C for 18 hr. After centrifugation (12,100 x g; 30 min; 4°C), the pellet was washed with 70% cold ethanol and vacuum dried. This was resuspended in a small volume of sterile H₂O.

Chromosomal DNA was prepared according to Ausubel *et al.* (9) with modifications. A culture of the *E. coli* strain of interest (1 liter) was grown to mid-log phase (3×10^8 CFU/ml). The cells were harvested by centrifugation at 2,300 x g, for 10 min at 4°C. The pellet was resuspended in 95 ml of TE buffer (10 mM Tris-HCl; 1 mM EDTA, pH 8.0).

Sodium dodecyl sulfate and proteinase K were added to a final concentration of 0.5% and 0.1 mg/ml, respectively. This was incubated for 1 hr at 37°C. The NaCl concentration of the sample was then adjusted to 0.7 M, mixed thoroughly and CTAB/NaCl solution (10% hexadecyl trimethylammonium bromide in 0.7 M NaCl) was added to a final concentration of 1% CTAB. This was heated to 65°C for 10 min, extracted with equal volume of chloroform: isoamyl alcohol (24:1) and extracted with phenol/chloroform/isoamyl alcohol (25:24:1). The DNA was precipitated with isopropanol and washed with 70% ethanol. The pellet was vacuum-dried, resuspended in TE buffer (7.5 ml) and centrifuged in a cesium chloride gradient (1 mg/ml cesium chloride; 0.625 mg/ml ethidium bromide) using similar parameters to the large scale plasmid preparation. Chromosomal DNA was removed from the gradient using a syringe fitted with a 16G x $\frac{1}{2}$ " needle and then extracted and precipitated as with plasmid DNA. Concentration was determined by agarose gel electrophoresis with known DNA standards and fluorometrically with Hoechst 33258 dye (using the TKO 100-dedicated Mini-Fluorometer; Hoeffer Scientific). Concentration as well as the purity of the DNA was determined from the absorption spectrum between the wavelengths 200 nm and 300 nm.

DNA sequence determination. DNA sequence was determined using the Sanger dideoxy method with double stranded plasmid DNA (85, 184). The plasmids used are listed in Table 2. Plasmids pSE-130, pSE-132 and pSE-133 used in the *fhlA* gene sequencing experiments were described

Table 2. Plasmids used in this study

Plasmid	Relevant Genotype or Phenotype	Reference
pRBH	Cm ^r , 14 kb <i>Cla</i> I- <i>Bam</i> HI fragment with the complete <i>hyd</i> -17 gene cluster from MC4100 in pACYC184	A. Bock (27)
pSE-111	Ap ^r , 14.7 kb <i>Sau</i> 3A1 fragment with the complete <i>hyd</i> (<i>hyp</i>) and partial <i>hyc</i> operon in pBR322	Laboratory collection (185)
pSE-125	Tc ^r , 2.8 kb <i>Sal</i> I fragment from pSE-22 with partial <i>hypC</i> and <i>fhIA</i> genes and complete <i>hypDE</i> (<i>hydFB</i>) genes in pBR322	Laboratory collection (187)
pSE-125-1	Tc ^r , 0.8 kb <i>Sal</i> I- <i>Cla</i> I fragment from pSE-125 with partial <i>hypCD</i> (<i>hydXF</i>) genes in pBR322	This study
pSE-128	Ap ^r , 6.5 kb <i>Sal</i> I- <i>Sau</i> 3A1 fragment from pSE-22 with partial <i>hypC</i> (<i>hydX</i>) gene and complete <i>hypDE</i> (<i>hydFB</i>) and <i>fhIA</i> genes in pBR322	Laboratory collection (185)
pSE-130	Ap ^r , 4.7 kb <i>Pst</i> I fragment with partial <i>hypA</i> and <i>fhIA</i> genes and complete <i>hypBCDE</i> (<i>hydEXFB</i>) genes in pBR322	Laboratory collection (186)
pSE-132	Ap ^r , 2.0 kb <i>Kpn</i> I- <i>Sal</i> I fragment from pSE-125 with partial <i>hypD</i> (<i>hydF</i>) and <i>fhIA</i> genes and complete <i>hypE</i> (<i>hydB</i>) gene in pUC19	Laboratory collection (187)
pSE-133	Ap ^r , 3.8 kb <i>Cla</i> I fragment with partial <i>hydF</i> gene and complete <i>hydB</i> and <i>fhIA</i> genes	Laboratory collection (187)
pSE-133-1	As pSE-133, <i>hydB</i> ::Tn5	This study
pSE-133-2	As pSE-133, <i>fhIA</i> ::Tn5	This study
pSE-137	Ap ^r , 1.0 kb <i>Kpn</i> I- <i>Sal</i> I fragment from with partial <i>hypCD</i> (<i>hydXF</i>) genes in pUC19	Laboratory collection (138)
pSE-190	Ap ^r , 1.3 kb <i>Sal</i> I- <i>Pst</i> I fragment from pSE-111 with partial <i>fhIA</i> gene in pUC19	Laboratory collection (188)

Table 2. continued

Plasmid	Relevant Genotype or Phenotype	Reference
pSE-1009	Ap ^r , 2.9 kb <i>KpnI</i> - <i>EcoRV</i> fragment from pSE-1007, complementing <i>molR</i> and <i>chlD</i> mutants, in pUC19	Laboratory collection (136)
pSE-1009 Exo #1-13	Ap ^r , <i>ExoIII</i> deletions from <i>KpnI</i> , in pUC19	This study
pSE-1004	Ap ^r , 4.7 kb <i>PvuII</i> - <i>ClaI</i> fragment from pSE-1001, complementing <i>molR</i> and <i>chlD</i> mutants, in pBR322	Laboratory collection (136)
pSE-1213	Ap ^r , 6.5 kb <i>Sau3A1</i> fragment with partial <i>hyb</i> operon in pBR-322	Laboratory collection
33pBR	Tc ^r , 6.2 kb <i>PstI</i> - <i>EcoRI</i> fragment with complete <i>hyb</i> operon	A. E. Przybyla
pSV208	Ap ^r , Cm ^r , <i>EcoRI</i> - <i>BamHI</i> promoter fragment from pBN208 [Ap ^r , Φ (<i>fdhF'</i> -' <i>lacZ'</i>) <i>hyb39</i> , 240 bp upstream of <i>fdhF</i>] into pKK232-8	A. Bock (196)

previously (187, 188). Plasmid pSE-190 carries a 1.0 kb *SalI*-*PstI* internal fragment of *fhIA* gene from previously described plasmid pSE-128 (187) in vector plasmid pUC19. Sequencing of the *hydX'*FB (*hypC'*DE) genes, upstream of the *fhIA* gene, was accomplished using plasmid pSE-137, a *SalI*-*KpnI* fragment containing the partial *hydX* (*hypC*) and *hydF* (*hypD*) genes in vector plasmid pUC19, pSE-132 which was described previously, and plasmid pSE-125-1 which was constructed as a 2.38 kb *ClaI* deletion of previously described plasmid pSE-125 (185). The *mol* operon was sequenced using previously described plasmid pSE-1009 (134, 136) and exonuclease III-generated deletion derivatives from the *KpnI* (the 5'-end of the open reading frames) towards the *EcoRV* site (88). Procedures from Promega "Erase-a-base system" technical manual were followed in these experiments. Plasmid pSE-1004 was also used to establish the *EcoRV* to *ClaI* DNA sequence. Both strands of chromosomal DNA present in these plasmids were sequenced using appropriate primers. New oligonucleotide primers were synthesized, as needed, based on the partial DNA sequence of the genes, by the DNA synthesis core laboratory, Interdisciplinary Center for Biotechnology Research, and by Dr. F.C. Davis, Department of Microbiology and Cell Science, University of Florida. Commercially available sequencing primers were obtained from US Biochemical Corporation, Pharmacia-LKB or New England BioLabs. DNA sequence was determined using T7 DNA polymerase (Sequenase), obtained from either US Biochemical Corporation or Pharmacia-LKB, and [³⁵S]-dATP was supplied by DuPont-New England Nuclear. The protocols supplied by

the manufacturers were followed. The DNA sequence was manipulated and homology with other known sequences in the Genbank and EMBL library was determined using the computer programs provided by Genetics Computer Group, University of Wisconsin (56, 234) and Genepro (Hoeffer Scientific).

DNA sequencing gels (both 21 x 50 cm and 38 x 50 cm) were poured and run according to the procedures supplied by BioRad Laboratories with modifications. A 6% polyacrylamide gel (acrylamide:bis 19:1) in 1 X TBE (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA) with 8 M urea was used to separate the reaction products. For increasing the length of DNA sequence determined, a variety of successful techniques were implemented. These included the use of wedge spacers provided by BioRad laboratories, "double loading", and the addition of sodium acetate (final concentration of approximately 0.1 M) to the bottom chamber in order to form an electrolyte gradient (201).

Southern transfer and hybridization. Restriction endonuclease digested chromosomal DNA was separated by electrophoresis in a vertical 1.0% agarose (Sigma type 1: low electroendosmosis grade) gel. The DNA was stained with ethidium bromide and photographed for calculating the fragment size of the hybridizing fragments. The DNA was depurinated (0.25 M HCl for 10 min) and denatured (0.5 N NaOH; 1 M NaCl for 30 min), and then the gel was neutralized (0.5 M Tris-HCl pH 7.4; 3 M NaCl for 30 min). Southern transfer to Zeta-Probe membranes (Bio-Rad Laboratories) was implemented using standard blotting techniques with 10 X SSC (1.5 M

NaCl; 0.15 M trisodium citrate) buffer (150). Membranes were dried under vacuum at 80°C for 3 hr and stored dry between two pieces of "Whatman" 3MM filter paper in plastic bags until used for hybridization. DNA probes were labeled by random primed incorporation of digoxigenin-labeled deoxyuridine-triphosphate (Dig-dUTP), hybridized under stringent conditions to the transferred DNA, and immunologically detected using an alkaline phosphatase linked antibody-conjugate. Procedures provided with the Boehringer-Mannheim Biochemicals "Genius"-kit were performed to carry out these experiments.

For localization of the *lac* fusion in strain SE-2007, an internal *fhla* gene fragment (1.3 kb *SalI*-*PstI*) from plasmid pSE-190 was labeled and used to probe chromosomal DNA from strains SE-2007 and MC4100 (parent) digested with either a single or two restriction endonucleases (*SalI* and *BglI*, *SalI* and *EcoRI*, *SalI* and *ClaI*, or *ClaI*). Similarly *lac* fusion strain SE-2001 (strains MC-4100 and SE-2009 were used as controls) chromosomal DNA was digested with endonucleases *KpnI* and *SalI*, *ClaI* and *BglI*, *BglI*, or *SalI* and *EcoRI* and probed with a 0.7 kb *SalI*-*KpnI* fragment from plasmid pSE-137 (carrying the partial *hypCD* genes). Further localization of the *lac* fusion was done on both mutant strains, SE-2001 and SE-2002, by digesting chromosomal DNA with *PstI* and then using 4.0 kb and 4.6 kb *PstI* fragments from plasmid pSE-111 as probes.

RESULTS AND DISCUSSION

Physiological Properties of an *fhlB* Mutant, Strain SE-2011

Biochemical characteristics of an *fhlB* mutant, strain SE-2011.

Using λ placMu53 mutagenesis (29), strain SE-2011 was isolated as a *lac* operon fusion derivative of strain MC4100 which produced β -galactosidase activity anaerobically and was deficient in fermentative dihydrogen production (Fhl^-). Upon detailed biochemical analysis, this strain was found to be affected in the production of hydrogenase, formate dehydrogenase-H, and fumarate reductase activities (Table 3). The lack of tritium exchange activity in strain SE-2011 shows that all three HYD isoenzymes are absent in this strain. As a consequence of this defect, both FHL, which requires active HYD-3, and hydrogen uptake, mediated by HYD-2, activities were not detectable in this strain. The FDH-H activity in strain SE-2011 was less than 10% of the levels observed in the parent, strain MC4100. On the basis of this property, strain SE-2011 can be distinguished from all known *hyd* mutants, which produced FDH-H activity. Similarly, the SE-2011 phenotype can be readily distinguished from FDH-H mutants using the hydrogen uptake characteristics of the other strains. In experiments which are similar to the ones described in Table 3, strain M9s, a known *fdhF* mutant (171), produced 75 to 85% of hydrogen uptake activity of the parent, strain

Table 3. Biochemical characteristics of strain MC4100 and an *fhbB* mutant, strain SE-2011

Enzyme	Specific activity	
	MC4100 (parent)	SE-2011 $\Phi(fhbB'-lacZ^+)$
Hydrogenase ^a (³ H ₂ -exchange)	1,200	44
Hydrogen uptake (H ₂ to BV) ^b	700	29
(H ₂ to fumarate) ^c	290	UD ^g
Formate hydrogenlyase ^d	170	UD
Formate dehydrogenase-H ^e	490	38
Fumarate reductase ^f	600	100

^aExpressed as nanomoles of ³H₂O produced per minute per milligram of cell protein.

^bExpressed as nanomoles of BV reduced per minute per milligram of cell protein.

^cExpressed as nanomoles of H₂ consumed per minute per milligram of cell protein.

^dExpressed as nanomoles of H₂ produced per minute per milligram of cell protein.

^eExpressed as nanomoles of BV reduced per minute per milligram of cell protein.

^fExpressed as nanomoles of BV_{red} oxidized per minute per milligram of cell protein.

^gUD, Undetectable

MC4100, measured either as BV or fumarate reduction. The hydrogenase activity of this strain, measured as tritium exchange was close to 100% of the parent. Strain M9s produced elevated levels of FDH-N (6.6-fold) and lower levels of fumarate reductase (28%) as compared with its parent, strain MC4100. These values which are comparable to the phenotype described by Pecher *et al.* (171) are quite distinct from the properties of strain SE-2011 (Table 3).

Strain SE-2011 is normal for nitrate respiration which requires active FDH-N, but has not been tested for the recently described third FDH isoenzyme which is presumed to be a major component of formate oxidase, expressed both aerobically and anaerobically (192). The level of fumarate reductase activity in strain SE-2011 was also lower (less than 20% of the parent value). This deficiency is probably the reason for the growth characteristics of strain SE-2011. The aerobic and anaerobic growth of this mutant was comparable to that of the parent, strain MC4100, in LB supplemented with different sugars. However, strain SE-2011 failed to grow in glucose-minimal medium and to produce succinate as a fermentation product. Since succinate is a necessary precursor for biosynthesis, this would account for the poor growth of the organism in defined medium. As will be discussed later, the same phenotype was observed when this mutation was transduced into strain MC4100 or other *lac* deletion mutants of *E. coli* (strains CSH26 and BW545), indicating that the pleiotropic effect is due to a single gene defect in these genetic backgrounds. The altered gene is termed *fhlB*

since the gene is formate inducible (see below) and thus probably plays a major role in the production of FHL activity. However, defects in the production of all three HYD isoenzymes and FR can be readily detected in strain SE-2011.

Formate requirement for expression of the *fhlB* gene. In order to better understand the transcriptional control of the FHL pathway, expression of the *fhlB* gene was monitored by measuring the levels of β -galactosidase activity produced by strain SE-2011 from the *fhlB* promoter. When cultured under strictly aerobic conditions, strain SE-2011 produced about 100 U of β -galactosidase activity in all media tested (Table 4). Upon transfer to anaerobic conditions, the β -galactosidase activity of the LB culture increased approximately 2.5-fold after a 4 hr incubation period. Fumarate had no effect on this anaerobic induction. Nitrate, a high redox potential electron acceptor, had a repressive effect on anaerobic expression. Both glucose and formate supplementation elevated *fhlB* expression anaerobically by about 2-fold and 5-fold, respectively. The glucose enhancement was due to the endogenous production of formate which was verified (see below) by the lack of glucose induction in a *pfl* background, deficient in pyruvate formate lyase activity. Acidic pH has been shown in the past to increase FHL activity (17, 78). Formate-dependent induction was reduced by 50% when the culture medium was buffered at pH 7.0. This parallels the physiological data which suggests a role for FHL in pH stabilization during the fermentative growth of *E. coli*.

Table 4. The effect of media composition on the expression of $\Phi(fhlB'-lacZ^+)$ in an *fhlB* mutant, strain SE-2011

Medium composition	β -Galactosidase activity ^a	
	Aerobic	Anaerobic
Luria Broth	110	280
+ Nitrate (1.0%)	90	120
+ Fumarate (0.5%)	110	250
+ Glucose (0.3%)	92	580
+ Formate (0.1%)	170	1,300
+ Formate and Glucose	190	1,400
+ Formate and buffer ^b	ND ^c	700

Cells were grown as described in the "Methods" section.

^aExpressed as nanomoles of o-nitrophenol produced per minute per milligram of protein.

^b0.1 M Phosphate buffer at pH 7.0

^cND, not determined

Upon detailed analysis of *fhlB* gene expression, monitored at specific time intervals, it was found that there was an exponential increase in β -galactosidase activity which paralleled the growth. The specific activity of the culture reached maximum value during early stationary phase and remained constant over an additional 8 hr of incubation (data not shown). In LB medium, maximum activity observed was about 250 U (Fig. 3). In this medium, the increase in specific activity of the enzyme was coupled to growth and the differential rate of induction was about 1.0. In LB medium supplemented with glucose, the differential rate of β -galactosidase production increased exponentially during growth, probably due to continued production of formate by the growing culture (since the amount of formate produced by the culture is proportional to the cell density). The maximum activity reached was about 600 units during the early stationary phase of growth when the cell density was about 100 μg protein/ml. With the addition of formate to the medium, the growth rate and final cell yield decreased, although the differential rate of synthesis of β -galactosidase was enhanced to as high as 130 units/ μg cell protein. In this medium, the maximum activity produced by the culture increased to about 1,200 units. In LB medium supplemented with both glucose and formate, the differential rate of induction of β -galactosidase activity was similar to the values obtained with LB-formate cultures but the total amount of the enzyme produced by strain SE-2011 was slightly higher (about 1,400 units). The final cell yield of the culture in the latter two media was comparable. These

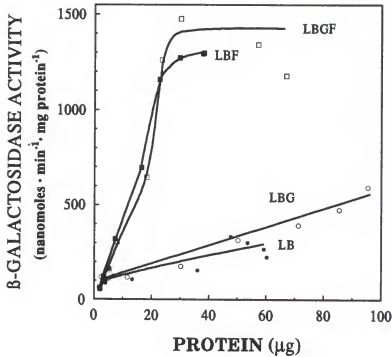


Figure 3. Differential rate of synthesis of β -galactosidase activity by $\Phi(fhlB'-'lacZ^+)$ strain SE-2011 grown in LB medium with different supplements. LBG, LBF, and LBGF represent LB-glucose, LB-formate, and LB-glucose-formate media, respectively. β -Galactosidase and protein activities are expressed as units per ml and μ g per ml, respectively.

experiments clearly show that the transcription of the *fhlB* operon is dependent on formate, either produced internally or added externally to the medium.

The amount of β -galactosidase activity produced by the $\Phi(fhlB'$ - $'lacZ^+$) strain increased linearly with increasing formate concentration up to about 5 mM (Fig. 4). The activity continued to increase at a lower rate until the maximum was reached at about 15 mM formate in the medium. For these experiments, strain SE-2011 was grown under anaerobic conditions, in LB-formate medium, and the cells were harvested after 4 hr for enzyme assays. Similar results were also obtained with strain MJ-9 [$\Phi(fhlB'$ - $'lacZ^+$), *pfl*] which lacks the ability to produce formate internally due to a loss of pyruvate formate lyase activity. At 15 mM formate, strain MJ-9 produced only about 60% of the β -galactosidase activity observed in the *pfl*⁺ parent strain. At higher formate concentrations (about 30 mM), the specific activity of β -galactosidase detected in strain MJ-9 was comparable to the *pfl*⁺ strain. These results suggest that the internally produced formate plays a significant role in the transcription of the *fhlB* operon.

Nitrate repression of *fhlB* gene expression. Further analysis of the repressive effect of nitrate on *fhlB* operon expression, was carried out by monitoring the β -galactosidase activity and nitrite produced by strain SE-2011 over a 6 hr growth period in an anaerobic environment. The *fhlB* mutant was grown in LB, LB-formate, LB-nitrate and LB-formate-nitrate medium (LB, LBF, LBN and LBFN respectively; Fig. 5). Nitrite

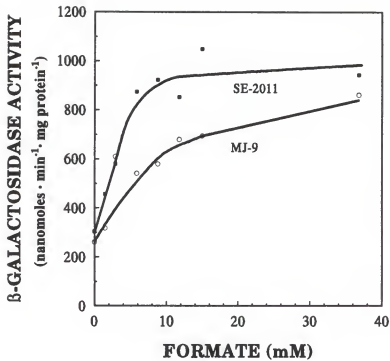


Figure 4. Effect of formate on the induction of β -galactosidase activity by Φ (*fhlB-lacZ* $^{+}$) strain SE-2011 and a *pfl* derivative, strain MJ-9. Specific activity represents the maximum value observed at each formate concentration.

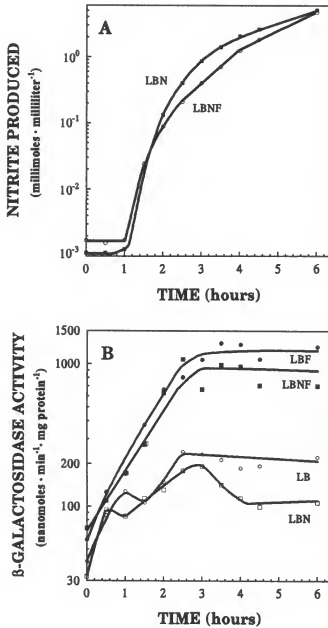


Figure 5. Effect of nitrate supplementation on *fhlB* gene expression. Cultures were grown in LB, LB-formate, LB-nitrate, and LB-nitrate-formate media (LB, LBF, LBN and LBNF, respectively). A. Nitrate respiration (as measured by nitrite produced) in *fhlB* mutant strain SE-2011 grown in LB and LBNF. B. Rate of induction of β -galactosidase activity by $\phi(fhlB^+ - lacZ^+)$ strain SE-2011.

production was measured in the cultures supplemented with nitrate to estimate the rate of nitrate respiration. Results of these experiments indicate that significant levels of nitrate respiration are not evident until 2 hr after initiation of anaerobiosis and growth (Fig. 5A). After this initial lag, nitrite accumulated in the medium throughout the remaining time of the experiment. In the absence of exogenous formate, nitrate repression of *fhlB* gene transcription (as measured by β -galactosidase activity) paralleled the initiation of nitrate respiration (LBN; Fig. 5B). Formate supplementation (73 mM) partially suppressed this repressive effect, and a *narL* mutation did not affect the *fhlB* operon regulation. This is comparable to the regulatory patterns observed for other genes of the FHL pathway (*hyd-17* and *fdhF* genes; 171). These results suggest that both formate and anaerobiosis are necessary for maximal induction of *fhlB* operon transcription. Although nitrate and neutral pH repress this expression, this effect can be overcome by formate.

Genetic Characteristics of Strain SE-2011, $\Phi(fhlB^{-}-'lacZ^{+})$

F' complementation analysis. Initial complementation studies were performed using F'143-1, an episomal element which carried DNA from 57 to 61 min in the *E. coli* chromosome. This was done since the previously described *hyd* and *fhlA* genes, which demonstrated pleiotropic phenotypes for dihydrogen metabolism, mapped to the 58 to 59 min region (185, 186, 187, 188). Mutant strain MJ-1, a *cys::Tn10* derivative of SE-2011, was used as a recipient for F'143-1 transfer. Exconjugants were selected for Cys⁺ phenotype and then screened for FHL activity. Approximately 20% of the Cys⁺ clones were Fhl⁺. Among those complemented for dihydrogen production, HUP activity was not restored (as measured by H₂ to BV). It is possible that the 20% complementation for FHL activity was because the entire region necessary for restoration of strain SE-2011 to parental phenotype was not completely transferred or that deletions may have occurred in the plasmid during growth with uracil, a needed nutrient. Therefore, strain MJ-50 [$\Phi(fhlB^{-}-'lacZ^{+})$ *cys*, *srl*] was used to select a F' element carrying the entire region between *cys*⁺ and *srl*⁺ genes. A Fhl⁺ exconjugant (5% of the clones tested) was isolated and used as a F'143-1 donor strain to select for Cys⁺ in recipient strain MJ-1, $\Phi(fhlB^{-}-'lacZ^{+})$ *cys*. Of the exconjugants analyzed, 100% were Fhl⁺ and Hup⁺ which suggested that the mutation mapped in this region.

P1 transduction. Similar results were obtained with P1 transduction experiments using strain BW545 as the donor. Of the 72

Cys⁺ clones tested, 12.5% were restored for FHL activity. One lysogen from this experiment tested positive for HUP activity but the clone cured of the lysogenic phage was deficient in both FHL and HUP activities.

Hfr mediated conjugation analysis of the *fhlB* gene. Because of these inconclusive results the approximate map location of the altered gene in the *E. coli* chromosome was determined by Hfr-mediated conjugation analysis. For these experiments, an Hfr-derivative of strain SE-2011 in which the origin of DNA transfer is the *fhlB* gene was constructed, using *lac* homology, as described before (40, 136). In a 30 min conjugation period, strain MJ-18, transferred *argI* gene (96.6 min; 11) and not *frdA* gene (94.4 min; 11) indicating that the *fhlB* gene was located between these two genes. Both *thr* and *leu* were also transferred at high frequency during this 30 min duration. Since the orientation of the *lac* operon with respect to the origin of transfer is known (40), results of these experiments were also used to determine the direction of transcription of the *fhlB* gene. According to the results, it appeared that the *fhlB* gene was transcribed in a clockwise direction, towards *argI*, *thr* and *leu*.

The accuracy of utilizing this procedure in certain *lac* fusion strains is currently under investigation (Shanmugam, personal communication). Therefore after reevaluating the physiological data and mapping results of SE-2011, it appeared probable that the localization of *fhlB* gene to the 96.6 min position was due to homology of the

chromosomal region (i.e. *melAB* at 93 min) to the episomal *lac* DNA sequences used in the construction of the Hfr (PO-*fhlB*). Comparable mapping complications were encountered in another study with strain SE-1100, a $\Phi(\textit{molR}'\text{'-}\textit{lacZ}^+)$ fusion. The *molR* gene was originally mapped to 66 min using similar Hfr procedures. Phage P1-mediated transduction experiments later mapped the *molR* gene at 17 min in the *E. coli* chromosome. Interestingly, a second ancestral gene for β -galactosidase, the *ebgA* gene, which is homologous to the *lacZ* gene was found to map in this region (68 min; ref. 8, 11, 32, 225, 226, 230).

Stability of the $\Phi(\textit{fhlB}'\text{'-}\textit{lacZ}^+)$ mutation. The pleiotropic nature of the *fhlB* mutation led to the question of whether there was one (or multiple) chromosomal mutation(s) leading to the observed phenotype in strain SE-2011. Initially, the same biochemical characteristics as well as operon expression (β -galactosidase activity) were observed when the mutation from strain SE-2011 was transduced into various *E. coli* strains, including strains MC4100, BW545 and CSH26. Even upon several years of maintaining the mutant strains at -70°C in 20% glycerol, the original characteristics of strain SE-2011 remained stable. However, in recent experiments, the majority of transductants which were selected for Lac^+ (X-gal^+) and Km^r were no longer analogous to the *fhlB* mutation previously described. Only about 1 to 5% of the transductants were Fhl^- . Dihydrogen uptake activity of these strains was comparable to wild type levels of activity. Many of these mutants were also altered in *fhlB* operon expression. Basal level anaerobic expression, in the

absence of formate was increased 2- to 3-fold. The significance of these results remains to be determined.

Plasmid complementation. Previously described plasmid pSE-133, which carries the partial *hydF* and complete *hydB*, *fhlA* genes (186), restored FHL activity of strain SE-2011 to low levels (data not shown). As later experiments would show, this effect is due to a physiological effect of the FHL-A protein, a transcriptional activator for the FHL system.

It is now known that the two divergently transcribed operons in the 58 to 59 min region (the *hyc* and *hyp* operons) span approximately 15.5 kb of DNA. Plasmid pRBH, which carries the complete *hyc* operon on low copy vector pACYC184 restored the Fhl^+ activity of strain SE-2011 to parental levels. Although the H_2 to BV activity was increased, this activity was attributable to HYD-3 (Table 5). Additionally, the β -galactosidase activity produced by strain SE-2011/pRBH was 2-fold lower than strain SE-2011 (LB-formate; 10 mM). Lutz *et al.* (144) have recently reported that the first gene of the *hyc* operon is a putative repressor and this is probably responsible for this decrease in *fhlB* expression.

Interestingly, multicopy plasmid pSE-1213 which carries the partial *hyb* operon (encoding HYD-2) and complements *hyb* mutants restored strain SE-2011 to parental levels of HUP and low levels of FHL activity (10% of wild type levels). Multicopy plasmid 33pBR which carries the complete *hyb* operon complemented strain SE-2011 for both FHL and HUP

Table 5. Plasmid complementation analysis of *fhlB* mutant, strain SE-2011

Strain/plasmid	Specific activity		
	FHL ^a	HUP ^b (H ₂ to BV)	HUP ^c (H ₂ to fumarate)
BW545	100%	694	44
SE-2011	UD ^d	UD	UD
SE-2011/pRBH	100%	+ ^e	ND ^f
SE-2011/pSE-1213	10%	1,017	63
SE-2011/33pBR	75%	616	91

^aExpressed as % wild type levels of H₂ produced.

^bExpressed as nanomoles of BV reduced per minute per milligram of cell protein.

^cExpressed as nanomoles of H₂ consumed per minute per milligram of cell protein.

^dUD, Undetectable.

^eMainly HYD-3 activity.

^fND, Not determined.

activities to 75% and 100% of wild type levels, respectively. This suggests that the mutation is more complex than anticipated. Further analysis is currently being done to determine the extent of the *hyb* operon on the *Sau3A1* fragment which is carried by plasmid pSE-1213. Also, the exact location of the *fh1B* mutation in strain SE-2011 is under investigation using polymerase chain reaction (PCR) procedures combined with $\Phi(fh1B^{-}-'lacZ^{+})$ cloning methods.

Trans-acting Factors of the *fhbB* Operon

Genetic regulation of the *fhbB* operon. Expression of the enzymes in dihydrogen metabolism requires the products of several genes and these include *hyd*, *fnr*, *fhbA*, *rpoN*, *molR* and *chbD* gene products (25, 35, 135, 136, 171, 185, 186, 187, 188, 196, 223, 224, 231, 238, 242). In order to study the role of these gene products on the expression of $\Phi(\textit{fhbB}'\text{-}'\textit{lacZ}^+)$, appropriate double mutant strains were constructed in which one of the putative regulatory genes is defective. Analysis of these double mutant strains revealed that the *fhbA*, *rpoN*, *chbD* and *molR* gene products are needed for the anaerobic formate-dependent induction of $\Phi(\textit{fhbB}'\text{-}'\textit{lacZ}^+)$ (Table 6). The differential rate of expression of β -galactosidase activity in the double mutants, strains MJ-5, MJ-20 and MJ-40 (*rpoN*, *fhbA* and *molR*, respectively) was unity indicating that the induction and cell growth are coupled and the enhancing effect of formate was absent. In an *fnr* mutant (strain MJ-6), although the differential rate of expression was comparable to strain SE-2011, the maximum activity was reduced by about 35%. The reduction in the amount of β -galactosidase activity produced by the *fhbB*, *fnr* double mutant could be a consequence of lower cell yield of the culture since the production of β -galactosidase activity by $\Phi(\textit{fhbB}'\text{-}'\textit{lacZ}^+)$ required growth of the organism. *HydF* and *cya* mutations had no apparent effect on the expression of $\Phi(\textit{fhbB}'\text{-}'\textit{lacZ}^+)$ operon with and without formate supplementation. Introducing *narL::Tn10* (217) into strain

Table 6. Expression of $\Phi(fhlB^+-lacZ^+)$ in different genetic backgrounds

Strain	Relevant Genotype	β -Galactosidase Activity ^a	
		Differential Rate of Induction ^b (U/ μ g Protein)	Maximum Activity
SE-2011	$\Phi(fhlB-lacZ^+)$	130	1,300
MJ-5	$\Phi(fhlB-lacZ^+)$, <i>rpoN</i>	1	110
MJ-20	$\Phi(fhlB-lacZ^+)$, <i>fhlA</i>	1	150
MJ-2	$\Phi(fhlB-lacZ^+)$, <i>hydF</i>	ND ^c	1,200
MJ-6	$\Phi(fhlB-lacZ^+)$, <i>fnr</i>	110	840
MJ-8	$\Phi(fhlB-lacZ^+)$, <i>cya</i>	160 ^d	1,300
MJ-40	$\Phi(fhlB-lacZ^+)$, <i>molR</i>	1	130
SE-1714	$\Phi(fhlB-lacZ^+)$, <i>chlD</i>	ND	160

^aExpressed as nmoles ONP produced per min per mg protein. All cultures were grown anaerobically at 37°C in LB + formate medium except strain MJ-6 which was grown in LB medium with formate and glucose to enhance cell yield.

^bThe differential rate of induction of β -galactosidase activity was calculated as the amount of enzyme activity produced by the culture in relation to the increase in total cell protein.

^cND, Not Determined.

^dPoor cell growth.

SE-2011 did not alter the amount of β -galactosidase activity produced by the culture both in the presence and absence of nitrate (data not shown).

Effect of multiple copies of the *fhlA* gene on *fhlB* transcription.

The effect of increasing the copy number of the *fhlA* gene on the expression of $\Phi(fhlB'-'lacZ^+)$ was investigated using plasmid pSE-133 which carries the complete *fhlA*⁺ and *hydB*⁺ genes (186, 187). In the presence of plasmid pSE-133, the differential rate of production of β -galactosidase activity by $\Phi(fhlB'-'lacZ^+)$ was about 700 units / μ g cell protein in LB-formate medium (Fig. 6). This value is greater than 5 times the rate of about 130 units / μ g cell protein for strain SE-2011 cultured in the same medium. The maximum activity produced by strain SE-2011/pSE-133 is also greater than 2 times the values obtained with strain SE-2011 and this increase was detected immediately after establishing anaerobic conditions. The maximum activity observed in strain SE-2011/pSE-133, grown in LB medium without formate supplementation, was also increased to about 1,100 units of β -galactosidase activity. This level of activity is comparable to the values obtained with strain SE-2011 grown in LB medium with 30 mM formate (about 1,000 units) although the differential rate of induction observed with strain SE-2011/pSE-133, in LB medium, was lower. Inserting transposon Tn5 into the *fhlA* gene in the plasmid (pSE-133-2; Fig. 7) abolished this enhancing effect of plasmid pSE-133 while a *hydB::Tn5* mutation in the plasmid (pSE-133-1) had no effect indicating that the plasmid-mediated increase is due to the *fhlA* gene. Transferring

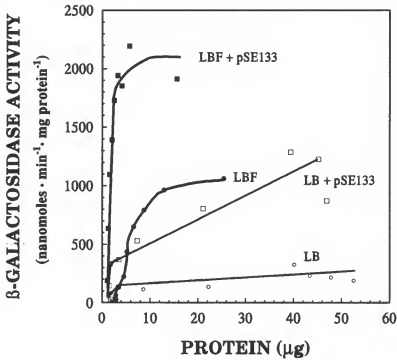


Figure 6. Differential rate of induction of the $\Phi(fhlB'-lacZ^+)$ fusion in strain SE-2011 in the presence and absence of plasmid pSE-133. LB, LB medium; LBF, LB medium supplemented with 30 mM formate. β -Galactosidase and protein activities are expressed as units per ml and μ g per ml, respectively.

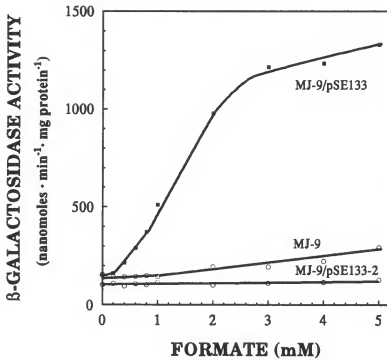


Figure 7. Effect of formate concentration on the levels of β -galactosidase activity produced by a $\Phi(fhlB'-'lacZ^+)$ *pfl* double mutant, strain MJ-9, in the presence of plasmids pSE-133 and pSE133-2 (*fhlA::Tn5*). Cultures were grown for 4 hr in LB medium with appropriate concentrations of formate under anaerobic conditions before the assay.

an F' element carrying *fhlA*⁺ gene (F'143-1) into strain SE-2011, did not significantly alter the rate or the level of β -galactosidase activity. These results suggest that increasing the copy number of the *fhlA*⁺ gene either decreased the concentration of formate required for transcription of $\Phi(fhlB'$ -'*lacZ*⁺) or eliminated the need for formate.

Formate is required for FHL-A activation of the *fhlB* operon. In order to distinguish between the two possibilities, plasmid pSE-133 was transferred to strain MJ-9 [$\Phi(fhlB'$ -'*lacZ*⁺), *pfl*] and the amount of β -galactosidase activity produced by the culture was determined after culturing the cells in either LB or LB-formate medium. The strain MJ-9/pSE-133 produced about 100 units of β -galactosidase activity when grown in LB medium and about 1,300 units when grown in LB-formate medium (Fig. 7). The minimum amount of formate needed for this transcription was about 3 mM. At formate concentrations higher than 3 mM, the amount of β -galactosidase activity produced by strain MJ-9/pSE-133 increased slowly reaching a maximum value of about 1,500 units at about 30 mM. The amount of β -galactosidase activity produced by strain MJ-9/pSE-133-2 (*fhlA*::Tn5) was actually lower than strain MJ-9 itself and under the conditions used in these experiments, this activity never exceeded 300 units. These results show that in the presence of multiple copies of *fhlA* gene, the concentration of formate required for optimum expression of *fhlB* gene is considerably reduced but is still a needed inducer.

The FHL-B protein is cytoplasmic. With 3 mM formate in the medium, the differential rate of synthesis of β -galactosidase activity

by strain SE-2011 was enhanced from about 20 units/ μ g protein to about 330 units/ μ g protein if the *fh1A* gene was also present in a multicopy plasmid (pSE-133-1) (Fig. 8). Immediately after anaerobic conditions were established, the *fh1B*-mediated β -galactosidase activity increased to about 1,200 units within a generation time. During the second generation, this activity decreased to about 75% of the observed peak value. The β -galactosidase activity of the culture reached about 1,000 units and was maintained at that level. On the other hand, strain M9s [Φ (*fdhF*'-'*lacZ*⁺)] carries the *lac* fusion in FDH-H, a known membrane protein. With the same plasmid, this strain (M9s/pSE-133-1) produced β -galactosidase activity after an initial lag. The differential rate of synthesis was about 10% of the values of strain SE-2011/pSE-133-1 and was only about 3-fold higher than strain M9s without the plasmid. The levels of β -galactosidase activity produced by strains SE-2011 and M9s, both in the presence and absence of the plasmid pSE-133-1, was not altered by including Mo or Se to the LB medium, although the FDH-H is a Mo, Se-containing protein. The amount of formate needed for optimum expression of Φ (*fdhF*'-'*lacZ*⁺)/pSE-133-1 was about 3 mM also which is similar to that required for strain MJ-9/pSE-133. This rapid induction of *fh1B* gene in the presence of multiple copies of *fh1A*⁺ gene, which is in contrast to the *fdhF* gene, suggests that the FHL-B protein is cytoplasmic. Its synthesis is apparently not constrained by the availability of membrane proteins and membrane synthesis.

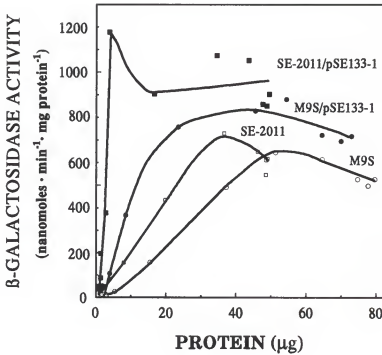


Figure 8. Differential rate of synthesis of $\Phi(fdhF^+ - lacZ^+)$, strain M9s, and $\Phi(fhlB^+ - lacZ^+)$, strain SE-2011, in LB medium supplemented with 3 mM formate in the presence and absence of the *fhlA*⁺ gene in a multicopy plasmid (pSE-133-1). β -Galactosidase and protein activities are expressed as units per ml and μ g per ml, respectively.

Expression of $\Phi(fdhF'$ - $'cat$) in an $fhlB$ mutant, strain SE-2011.

The FDH-H activity was undetectable in strain SE-2011. In order to determine whether this effect is at the transcriptional or post-transcriptional level, a plasmid carrying a 'CAT' gene fusion in the $fdhF$ gene was transformed into strain SE-2011 and the effect of the $fhlB$ mutation on $fdhF$ gene mediated CAT activity was determined. Cells were grown anaerobically (4 hr) in LB medium supplemented with glucose and formate for maximal expression. Due to the multicopy nature of the expression vector, trace metals (Se, Mo, and Fe) were included to insure saturation. The $fdhF$ transcription measured as CAT activity in strain SE-2011 [$\Phi(fhlB'$ - $'lacZ^+$)] was comparable to parent strain MC-4100 at 140 and 110, units respectively. Whereas, in strain SE-2007 [$\Phi(fhlA'$ - $'lacZ^+)$] expression was reduced over 5-fold, to about 20 units confirming the need for FHL-A protein. This suggests that FHL-B protein is not required for transcription of $fdhF$ gene but is required for the production of active FDH-H (Table 3).

Analysis of the *fhIA* Gene

Primary structure of the *fhIA* gene. Previous experiments identified the *fhIA* gene product as a putative regulatory element of both *fdhF* and *hyd-17* genes (185, 187, 196). The results presented above (Table 6, Fig. 6-8) also show that the FHL-A protein is a needed regulatory element for the *fhIB* operon. Because of these observations, the DNA sequence of the *fhIA* gene was determined to identify the characteristics of the gene and its product. The *fhIA* gene (coding region position 2421 to 4497; Fig. 9) codes for a protein of 692 amino acids with an anhydrous molecular weight of 78,467 Da which is comparable to the apparent molecular weight of 78,000 Da obtained by other experiments (144, 185). This protein did not contain any significant hydrophobic region indicating that the primary location of this protein is the cytoplasm. Eight base pairs from the end of translational stop codon (position 4497), the coding region is followed by an inverted repeat (underlined in Fig. 9; positions 4508 to 4517 and 4524 to 4533) which can produce a 10 base pairs stem and a 6 bases (positions 4518 to 4523) loop structure. This region is followed by a stretch of 6 thymine residues at a distance of 14 bases (positions 4548 to 4553). Five more thymine residues can be found 9 bases from the first set of thymines (positions 4563 to 4567). This segment of DNA, in appropriate configuration may function as a ρ -independent transcription termination site. The 5'-end of the putative coding region is preceded

SalI

GTCGACGCTCGCGCATTCAGCGCGATGTCGATTTAACGTTAGTCGGCAGCTGCGATGAA 60
 V D V C G G I Q R D V D L T L V G S C D E
 AACGGTCAGCCCGCGCTGGGCCAGTGGGTACTGGTACACGTTGGCTTTGCCATGAGCGTA 120
 N G Q P R V G Q W V L V H V G F A M S V
 ATTAATGAAGCCGAAGCACGCGACACTCTCGACGCCCTTACAAAACATGTTTGACGTTGAG 180
 I N E A E A R D T L D A L Q N M F D V E
 CCGGATGTCGGCGCGCTGTTGTATGGCGAGGAAAAATAATGCGTTTTGTTGATGAATATC 240
 P D V G A L L Y G E E K ***

M R F V D E Y R

GCGCGCCGGAACAGGTGATGCAGTTAATTGAGCATCTGCGCGAACGTCCTCACATCTCT 300
 A P E Q V M Q L I E H L R E R A S H L S
 CTTACACCGCCGAACGCCCTCTGCGGATTATGGAAGTGTGTGGCGGTATACCCACGCTA 360
 Y T A E R P L R I M E V C G G H T H A I
 TCTTTAAATTCGGCCTCGACCACTGTCGCGGAAAAACGTTGAGTTTATCCACGGTCCGG 420
 F K F G L D Q L L P E N V E F I H G P G
 GGTGCCCGTGTGCGTACTGCGGATGGGTAGAATCGACACCTGCGTGGAGATTGCCAGCC 480
 C P V C V L P M G R I D T C V E I A S H
 ATCCGGAAGTCATCTTCTGTACCTTTGGCGACGCGATGCGCGTCCGCGGGAACAGGGAT 540
 P E V I F C T F G D A M R V P T G K Q G S
 CGTGTTTGACGCAAAAGCACGCGGTGCCGATGTCGCGATCGTTTACTCGCCGATGCGATG 600
 L L Q A K A R G A D V R I V Y S P M D A
 CGTTGAAACTGGCGCAGGAGAATCCAACCCGCAAGTGGTGTCTTCGCGTTAGGTTTTG 660
 L K L A Q E N P T R K V V F F G G L G F E
 AAACCACTATGCCACACCGCTATCACTCTGCAACAGGCGAAAGCGCGTGTGTCGAGA 720
 T T M P T T A I T L Q Q A K A R D V Q N
 ATTTTACTTCTCTGCCAGCATTACGCTTATCCCAGCTTGGCGAGTTTGTCTGGAAC 780
 F Y F F C Q H I T L I P T L R S L L E Q

ClaI

AGCCGGATAACGGTATCGATGCGTTCTCGCGCCGGGTACGTCAGTATGGTTATCGGCA 840
 P D N G I D A F L A P G H V S M V I G T
 CCGACGCTATAATTTTATCGCCAGCGATTTTCATCGTCCGCTGGTGGTTGCTGGATTG 900
 D A Y N F I A S D F H R P L V V A G F E
 AACCCCTTGATCTACTACAAGCGTGGTCATGCTGGTGCAGCAGAAAAATAGCGGCCACA 960
 P L D L L Q G V V M L V Q Q K I A A H S

KpnI

GCAAGGTAGAGATCAGTATCGTCGAGTGGTACCGGATGCCGGTAACCTGCTGGCGCAAC 1020
 K V E N Q Y R R V V P D A G N L L A Q Q

Figure 9. Nucleic acid and predicted amino acid sequences of the partial *hypC* gene and complete *hydB*, *hydF* and *fhlA* genes. The termination codons are indicated by three asterisks. The "Shine-Dalgarno" sequences and the weak "-35 and -10" region of the *fhlA* gene are double underlined. Restriction sites for some of the enzymes are highlighted. The inverted triangle between positions 4,404 and 4,405 represents the position of transposon Tn5 in plasmid pSE133-2, as determined by DNA sequence analysis.

AGGCGATTGCCGATGTGTTCTGTGTCAACGGCGACAGCGAATGGCGCGGCTTAGGCGTGA 1080
 A I A D V F C V N G D S E W R G L G V I
 TTGAATCTTCTGGCGTGACCTGACGCCGATTATCAACGATTCGATGCCGAAGCACATT 1140
 E S S G V H L T P D Y Q R F D A E A H F
 TCCGCCCGGACCGCAGCAGGTCTGCGATGACCCGCGCGCGTGTGGTGAGGTATTAA 1200
 R P A P Q Q V C D D P R A R C G E V L T
 CGGGCAAATGTAAGCCGATCAATGCCCGCTGTTTGGTAACACCTGTAATCCTCAAACCG 1260
 G K C K P H Q C P L F G N T C N P Q T A
 CGTTTGGTGCGCTGATGGTTTCTCCGAAGGAGCGTGCGCCGCGTGGTATCAGTATCGTC 1320
 F G A L M V S S E G A C A A W Y Q Y R Q
 AGCAGGAGAGTGAAGCGTGAATAATATCCAACCTCGCCACGGTAGCGGGCGCCAGGCGAT 1380
 Q E S E A *** M
 GCAGCAATTAATCAACAGCCTGTTTATGGAAGCCTTTGCCAACCCGTTGGCTGGCAGAGCA 1440
 Q Q L I N S L F M E A F A N P W L A E Q
 GGAAGATCAGGCACGCTTGTATCTGGCGCAGCTGGTAGCGGAAGGCGACCGTCTGGCGTT 1500
 I D Q A R L D L A Q L V A E G D K A F
 CTCACCACGAGTTACGTTATTGACCCGCTGTTCTTCCCTGGCGGTAATATCGGCAAGCT 1560
 S T D S Y V I D P L F F P G G N I G K L
 GGCAGTTTGGCGCACAGCAATGACGTTGCGGTGAGTGGCGCTATTCGCGCTATCTCTC 1620
 A I C G T A N D V A V S G A I P R Y L S
 CTGTGGCTTTATCCTCGAAGAAGGATTGCCGATGGAGACACTGAAAGCCGTAGTGACCAG 1680
 C G F I L E E G L P M E T L K A V V T S
 CATGGCAGAAACCGCCCGCGCGGCGAGGCATTGCCATCGTTACTGGCGATACTAAAGTGT 1740
 M A E T A R A A G I A I V T G D T K V S
 GCAGCGCGGCGCGGTAGATAAACTGTTTATCAACACCGCTGGCATGGGCGCAATTCCGGC 1800
 Q R G A V D K L F I N T A G M G A I P A
 GAATATTACTGGGGCGCACAGACGCTAACCAGCGGCGATGATTGCTGGTGAGCGGTAC 1860
 N I H W G A Q T L T A G D V L L V S G T
 ACTCGGCGACACGGGGCGACTATCCTTAACCTGCGTGAGCAGCTGGGGCTGGATGGCGA 1920
 L G D H G A T I L N L R E Q L G L D G E
 ACTGGTCAGCGACTGCGCGGTGCTGACGCCGCTTATTCAGACGCTGCGTGACATTCCCGG 1980
 L V S D C A V L T P L I Q T L R D I P G
 CGTGAAGACGCTGCGTATGCCACCCGTTGGTGGTGAACGCGGTGGTTCTAGATTCGC 2040
 V K A L R D A T R G G V N A V V H E F A
 GGCAGCTGCGGTTGTGGTATTGAACCTTCAGAAGCGGCACTGCCTGTTAAACCTGCCGT 2100
 A A C G C G I E L S E A A L P V K P A V
 GCGTGGCGTTTGCGAATGCGGACTGGACGCCCTGAACCTTGCCAACGAAGGCTTTCGCTTCG 2160
 R G V C E L L G L D A L N F A N E G K L
 AGTAATAGCTGTTGAACGCAACGCGGCAGAGCAAGTGTGCGCAGCGTTACATTCCCATCC 2220
 V I A V E R N A A E Q V L A A L H S H P
 ACTGGGGAAGACGCGGCGCTGATTGGTGAAGTGGTGAACGTAAGGTTTCGCTTCG 2280
 L G K D A A L I G E V V E R K G V R L A
 CGGTCTGTATGGCGTGAACGAACCTCGATTTACCACACGCCGAACCGCTTCCGCGTAT 2340
 G L Y G V K R T L D L P H A E P L P R I

Figure 9--continued.

ATGCTAATAAAATCTAAATCTCTATAGTTAGTCAATGACCTTTTGACCGCTTTGCGG	2400
C *****	
TGCTTTCTCGGAAGAACAATAATGTCATATACACCGATGAGTGATCTCGGACAACAAGGGT	2460
M S Y T P M S D L G Q Q G L	
TGTTGCGATCACTCGGACACTATTGCAGCAGCCGATCTGGCCTCGCTGTGTGAGGCTC	2520
F D I T R T L L Q Q P D L A S L C E A L	
TTTCGCAACTGGTAAAGCGTTCTGCGCTCGCCGACAACGCGGCTATTGTGTTGTGGCAAG	2580
S Q L V K R S A L A D N A A I V L W Q A	
CGCAGACTCAACGTGCGTCTTATTACGCGTCGCGTGAAAAAGACACCCCATTAATATG	2640
Q T Q R A S Y Y A S R E K D T P I K Y E	
AAGACGAAACTGTTCTGGCACACGGTCCGGTACGCAGCATTTGTGCGGCCCTGATACGC	2700
D E T V L A H G P V R S I L S R P D T L	
TGCATTGCAGTTACGAAGAATTTGTGAAACCTGGCCGACGTGGACGCAGGTGGGCTAT	2760
H C S Y E E F C E T W P Q L D A G G L Y	
ACCCAAATTTGGTCACTATTGCCTGATGCCACTGGCGGCGGAAGGGCATATTTTGGTG	2820
P K F G H Y C L M P L A A E G H I F G G	
GCTGTGAATTTATTCGTTATGACGATCGCCCTGGAGCGAAAAAGAGTTCAATCGTCTGC	2880
C E F I R Y D D R P W S E K E F N R L Q	
HpaI	
AAACATTACGCAGATCGTTTTCTGTCGTCACCGAACAATCCAGAGCCGCGTCGTTAAACA	2940
T F T Q I V S V V T E Q I Q S R V V N N	
SalI	
ATGTCGACTATGAGTTGTTATGCCGGGAACGCGATAACTTCCGCATCTGGTCGCCATCA	3000
V D Y E L L C R E R D N F R I L V A I T	
CCAACGCGGTGCTTTCCCGCTGGATATGGACGAACTGGTCAGCGAAGTCGCCAAAGAAA	3060
N A V L S R L D M D E L V S E V A K E I	
TCCATTACTATTTGCACTTACGATATCAGTATCGTCTTACGCAGCCACCGTAAAAACA	3120
H Y Y F D I D D I S I V L R S H R K N K	
AACTCAACATCTACTCCACTCACTATCTTGATAAACAGCATCCCGCCACGAACAGAGCG	3180
L N I Y S T H Y L D K Q H P A H E Q S E	
AAGTCGATGAAGCCGGAACCTCACCGAACGCGTGTTCAAAAGTAAAGAGATGCTGCTGA	3240
V D E A G T L T E R V F K S K E M L L I	
TCAATCTCACAGCGGGACGATTTAGCCCTATGAACGAGCATGTTGTGACACCTGGG	3300
N L H E R D D L A P Y E R M L F D T W G	
GCAACGAGATTCAAACCTGTGCGCTGTACCCTGATGTCTGGCGACACCATGCTGGGCG	3360
N Q I Q T L C L L P L M S G D T M L G V	
TGCTGAAACTGGCGCAATGCGAAGAGAAAGTGTTCACCACTACCAATCTGAATTTACTGC	3420
L K L A Q C E E K V F T T T N L N L L R	
GCCAGATTGCCGAACGTGTGGCAATCGCTGTCGATAACGCCCTCGCCTATCAGGAAATCC	3480
Q I A E R V A I A V D N A L A Y Q E I H	
ATCGTCTGAAAGAACGCGTGGTTGATGAAAACCTCGCCCTGACCGAGCAGCTCAACAATG	3540
R L K E R L V D E N L A L T E Q L N N V	
TTGATAGTGAATTTGGCGAGATTATTGCCCCAGCGAAGCCATGTACAGCGTGCTTAAAC	3600
D S E F G E I I G R S E A M Y S V L K Q	

Figure 9—continued.

AAGTTGAAATGGTGGCGCAAAGTGACAGTACCGTGCTGATCCTCGGTGAAACTGGCACGG	3660
V E M V A Q S D S T V L I L G E T G T G	
GTAAGAGCGTGATTGCCGTGCGATCCATAATCTCAGTGGCGTAATAATCGCCGCATGG	3720
R G A I T G A S A I H N L S G R N N R R M V	
TCAAATGAAGTGCAGCGCGATGCCTGCCGATTGCTGGAAAGCGATCTGTTTGGTCATG	3780
K M N C A A M P A G L L E S D L F G H E	
AGCGTGGGGCTTTTACCGTGCCAGCGCCAGCGTATCGGTGTTTGAAGTGGCGGATA	3840
R G A F T G A S A Q R I G R F E L A D K	
AAAGCTCCCTGTTCTCGACGAAGTGGCGATATGCCACTGGAGTTACAGCCGAAGTTGC	3900
S S L F L D E V G D M P L E L Q P K L L	
TGCGTGATTGCAGGAACAGGAGTTTGAACGTCTCGGCAGCAACAAATCATTAGACGG	3960
R V L Q E Q E F E R L G S N K I I Q T D	
ACGTGCGCTAATCGCCGCGACTAACCAGCATCTGAAAAAATGGTCGCCGACCGTGAGT	4020
V R L I A A T N R D L K K M V A D R E F	
TCCGTAGCGATCTCTATTACCGCTGAACGTATCCCGATTACCTGCCGCCACTACGCG	4080
R S D L Y Y R L N V F P I H L P I L R E	
AGCGTCCGGAAGATATTCCGCTGCTGGCGAAAGCCTTTACCTTCAAATTCGCCGTCGTC	4140
R P E D I P L L A K A F T F K I A R R L	
TGGGCGCAATATCGACAGCATTCTGCCGAGACGCTGCGCACCTTGAGCAACATGGAGT	4200
G R N I D S I P A E T L R T L S N M E W	
GGCCGGGTAACTACGCGAACTGGAAAACGTCATTGAGCGCGGATTGCTAACACGCG	4260
P G N V R E L E N V I E R A V L L T R G	
PstI	
GTAACGTGCTGAGCTGTCTATTGCCAGATATTGTTTTACCGAACCTGAAACGCCGCCCTG	4320
N V L Q L S L P D I V L P E P E T P P A	
CCGCAACGGTTGTCGCCCTGGAGGGCGAAGATGAATATCAGTTGATTGTGCGCGTGCTGA	4380
A T V V A L E G E D E Y Q L I V R V L K	
Tn5	
▼	
AAGAAACCAACGGCGTGGTTGCCGGGCCTAAAGGCGCTGCGCAACGTCTGGGGCTGAAAC	4440
E T N G V V A G P K G A A Q R L G L K R	
GCACGACCCTGCTGCACGGATGAAGCGGCTGGGAATTGATAATCGGCATTGATTTAAC	4500
T T L S R M K R L G I D K S A L I ***	
TGCAAAATGCCGGACAGATCTGCCTGTCCGGCATACTATTTCATGAGGTTTTTTCGGACGA	4560
ClaI	
TATTTTTCCGGCAGTTCTGGCACCGGACGCTTGTGCATCGAT	4601

Figure 9—continued.

by a typical ribosome binding site (GGA, starting at position 2410; ref. 126). A weak "-10 and -35" σ^{70} promoter consensus sequence is also indicated in Fig. 9 (positions 2312 to 2317 and 2338 to 2343; ref. 86). Based on the DNA sequence, the *fhlA* gene resides between 2,867 and 2,870 kb of the *E. coli* chromosomal DNA as described by Kohara *et al.* (125) and the direction of transcription is clockwise towards *cys* operon at 59 min (11).

FHL-A protein is a transcriptional activator. The FHL-A protein has sequence homology with known transcriptional activators like NTR-C protein of *E. coli*, NIF-A protein of *Klebsiella pneumoniae*, and XYL-R protein of *Pseudomonas putida* (Fig. 10; and ref. 58, 103, 157). The overall homology is about 30% between the four proteins although the FHL-A protein is considerably larger than the other three. It is 224, 168, and 126 amino acids larger than NTR-C, NIF-A and XYL-R proteins, respectively. Significant regions of the FHL-A protein were also found to be homologous with other transcriptional activators of the two-component regulatory systems including the recently described LEV-R protein of *Bacillus subtilis* (53), DCT-D protein of *Rhizobium leguminosarum* (181), FLB-D protein of *Caulobacter crescentus* (176), TYR-R protein (49) and HYD-G protein of *E. coli* (224). Homology with this class of proteins, combined with physiological data, suggest that the FHL-A protein functions as a transcriptional activator of the σ^{54} -dependent promoters of the FHL pathway (*fhlB*, *fdhF* and *hyc* operons; for review, see 80, 222).

FHL-A	MSYTPMSDLGQQLFDITRTLLQQPDLASLCEALSQVKKRSALADNAAIVLWQAQTQRAS	60	
FHL-A	YYASREKDTPIKYEDETVLAHGPPVRSILSRPDTLHCSYEEFCETWPQLDAGGLYPKFGHY	120	
FHL-A	CLMPAAEGHIFGGCEFIYDDRPWSEKEFNRLQTFQTIVSVVTEQIQSRVNNVDYELL	180	
NIF-A		MIHKSDDSTTV	11
XYL-R	MSLTYPKPKMQHEDMQDLSSQIRFVAEAGKIWL	33	
FHL-A	CRERDNFRILVAITNAVLSRLMDLSEVSEAKEIHYYFDIDDISIVLRSHRKNKLNIST	240	
NIF-A	RRFDLSQQTAMQRISVLSRATESKTLQEVLSVLHNDAFMQHGMICLYDSQQEILSIE	71	
XYL-R	EQRLMVQLSTLASFRREIISLIGVERAKGFRLRLGYQSGLMDAELARKLRPAMREEEVF	93	
FHL-A	HYLDKQHPAHEQSEVDEAGTLTERVFKSKEMLLINLHERDDLAPYERM-LFDTWGNQIQ	299	
NTR-C	MQRGIVVWDDSSIRWVLERALAGALTCTTFENGAEVLEA-LASKTPDVL	53	
NIF-A	ALQQTEDQTLPGSTQIRYRPGGLVGTVLAQGSQSLVLRVADDQRFLDRL-SLYDYDLPF	130	
XYL-R	LAPQLYALKGMVKVRLTMDIAIRDGRFNVEAEWIDSFEVDICRTELGL-MNEPVCWTV	152	
FHL-A	LCLPLMSGDTMLGVLKLAQCEEKVFTT-TNLLRQIA---ERVAIA-VDNALAYQEI	354	
NTR-C	DIRPMQDGLALKLQKQRHPVIMTA-HSDLDAAVSAYQQGAFIDYLPKPFIDYEL	112	
NIF-A	IAVPLMGPHSRPIGVIAAHAMARQEERLPA-CTRFLTV---ANLIAQ-TIRLMILPTSA	185	
XYL-R	LGYASGYGSFAFMGRRIIFQETSCRGGDDKCLIVGKTA---EEDGVSSFEAYFKSDPI-	208	
FHL-A	RLKER--LVDENLALTEQLN-NVDSEFGEIIGRSEAMYSVLKQVEMVAQSDSTVLIGET	411	
NTR-C	ALVER--AISHYQEQQQPRNVQLNGPTTDIIAKP-AMQDVFRIIGRLSRSSISVLINGES	169	
NIF-A	AQAPQ--QSPRIERPRACTP-SRGFGLENMVGKSPAMRQIMDIIRQVSRWDTTVLVRGES	242	
XYL-R	-VDREYELQTVANLNRNLK-QYDQYYG-IGHSPAYKRICETIDKAARGRVSVLLIGET	265	
FHL-A	GTGKELIARAIHNLGRNNRMVKMNCAMPAGLLESDFGHERGAFTGASAQIRGFEL	471	
NTR-C	GTGKELVAHALHRHSRAKAPFIALNMAAI PKDLIESELFGEKGAFTGANTIRQGRFQE	229	
NIF-A	GTGKELIANAIHNSPRAAAAFVKFNCAALPDNLLESELFGEKGAFTGAVRQRKGRFEL	302	
XYL-R	GVGKEVIARSVHLRSERAEQPFVAVNCAAIIPDLIESELFVGDKGAYTGAVNARAGRER	325	
FHL-A	ADKSLFLDEVGDMPLEQPKLLRLVQEDEFERLGSNKIQTVDRLIAATNRDLKMKVAD	531	
NTR-C	ADGGTFLDEIGDMPDLQVTRLLRVLDAGQFYRVGGYAPVVDVRIIAATHNLEQVRQE	289	
NIF-A	ADGGTFLDEIGESSASFQAKLLRILQEGEMERVGGDETLRVNVRIIAATHNLEEEVRL	362	
XYL-R	ANGGTIFLDEVIELTPRAQATLLRLVQEGELERVGGDRTRKVDVRLIATNENLEAAVKM	385	
FHL-A	REFRSDLYYRLNVFPIHLPPLRERPEDIPLAKAFTFKIARRLGRNIDSIPAETLRTLSN	591	
NTR-C	GKFRFDLHRLNVI RVHLPPLRERREDIPRLARHFLQVAARELGVEAKLLHPETEAALTR	349	
NIF-A	GHFRDLYYRLNVMPIALPPLRERQEDIAELAHFLVRKIAHSQGRTL-RISDGAIRLLME	421	
XYL-R	GRFRADLFFRLNVFPHIPLRERVEDIPLLVEHFLRRHKEYGKKTGLSDRAMEACLH	445	
FHL-A	MEWPGNVRELENVIERAVLLTRG-NVLQLSLPDI VLPE-PETPPAATVVALE-G--EDEY	646	
NTR-C	LAWPGNVRLQENTCRWLTVMAAGQEVLIQDLPGELFES-TVAESTSQMQPDSWA--TLA	406	
NIF-A	YSWPGNVRELENCLEARSVLES-GLIDRDVILFNHRDNPALKASSGPAED-G-----	473	
XYL-R	YQWPGNIRELENALERGVILTES-N--ESINVESLPG-LATATEGRDLSE-GRLEES	500	
FHL-A	QLIVRVLKETNGVVAG-----PKGAAQRLLGKRTTLLSRMKRLG	685	
NTR-C	QWADRALS GHQNLLESAQPELERTLLTALRHTQGHKOEAAARLLGWRNTLTTRKLELG	466	
NIF-A	-WLDNSLDERQRLIAALEKAGW-----QAKAARLLGHTPROVAYRIQIMD	518	
XYL-R	GDSW--FRQI--IDQGVSLLEDLEAGLMRTAMDRCGQNTSQAARLLGLTRPAMAYRLKLLD	556	
FHL-A	IDKSALI*	692	
NTR-C	ME*	468	
NIF-A	ITMPRL*	524	
XYL-R	PSLSVKAMGR*	566	

Figure 10. Alignment of the predicted sequences of *E. coli* FHL-A, *E.*

coli NTR-C, *K. pneumoniae* NIF-A, and *P. putida* XYL-R proteins.

Identical and functionally similar amino acids are highlighted.

Underlined regions represent those residues of which two out of the three aligned amino acids are homologous to FHL-A protein. The helix-turn-helix motif is double underlined.

Based on the sequence homology with these proteins, the FHL-A protein can be divided into 4 regions which are interspersed with segments of variable length. Region I is unique to FHL-A and includes the first 353 amino acids. Similarly, NIF-A, FLB-D, LEV-R and TYR-R are composed of a unique region at the amino-terminus. This singular region has been thoroughly analyzed in the NIF-A protein which is synthesized in an active form (21, 31, 63). In *K. pneumoniae*, the amino-terminus of the NIF-A protein is presumed to be the site of direct interaction with NIF-L (58). The NIF-L/NIF-A complex is inactive in the presence of O₂ and fixed nitrogen (93, 153).

The N-terminal domain of NTR-C and DCT-D, however, demonstrate significant homology to other pleiotropic control proteins which have been shown or are presumed to be phosphorylated by a sensor at an exposed aspartate residue (91, 101, 120, 161, 162, 240). The *S. typhimurium* CHE-Y protein is included in this class of phosphorylated regulators. The three-dimensional structure of purified CHE-Y protein has been resolved to 0.27 nm. It was found to be composed of a central hydrophobic core of five parallel β -sheets surrounded by five α -helices (221). Comparison of amino acid sequence suggested that this N-terminal secondary structure is conserved among the phosphorylated receivers. Stock *et al.* (222) reported that the highly conserved aspartate and lysine residues of these proteins are clustered at the carboxy-terminal end of the β -sheets 1,3 and 5. Furthermore, these conserved residues are the active site of the transcriptional regulator. Based on the

sequence, the FHL-A protein does not have this domain and thus may not undergo phosphorylation. The FHL-A region I probably determines the specific regulatory function for this protein in response to FHL inducers (i.e. formate and anaerobiosis).

The next region of about 24 amino acids, (region II, 356-374 amino acids) aligns with the proposed "interdomain linker" of both NTR-C and NIF-A proteins (58). The high content of glutamine (Q), serine (S), glutamic acid (E), arginine (R) and proline (P) residues, 55% in NTR-C and 74% in NIF-A, suggests a coil or turn conformation in the protein structure. This could hypothetically link the region in the N-terminus to the central ATP-binding region (discussed below). In FHL-A protein, the extent of these residues is apparently random (26%) suggesting an alternative secondary structure in this region.

The region III is about 240 amino acids (positions 380 to 619) and is highly conserved among transcriptional activators of the two-component regulatory systems which are proposed to interact at σ^{54} -dependent promoters (80). Alignment with FHL-A protein is presented in decreasing order of homology: NIF-A (53%), FLB-D (48%), NTR-C (47%), XYL-R (46%), DCT-D (43%), and TYR-R (39%; Fig. 11). This region is analogous to the "domain D" of the NIF-A and NTR-C proteins (58). The boxed residues are potential ATP-binding sites (53, 67, 229). Current evidence suggests that the activator protein binds to the σ^{54} -RNA polymerase holoenzyme via this domain and catalyzes the ATP-dependent isomerization of closed to open transcriptional complex (174). Non-

FHLA	EIIGRSEAMYSVLKQVEMVAQSDSTVLIL	GETGTGKE	LIARAIHNLSGRNNRMVVMNCA
NIFA	NMVGKSPAMRQIMDIIRQVSRWDTTVLVR	GESGTGKE	LIANAIHHNSPRAAAFVKFNCA
FLBD	PMVVRDPAMEQVVKILADQVAPSEASILIT	GESGSGKE	VMARYVHGKSRRAKAPFISVNCA
NTRC	DIIAK-PAMQDVFRIGRLSRSSISVLIN	GESGTGKE	LVAHALHRHSRAKAPFIALNMA
XYLR	G-IGHSPAYKRICETIDKAARGRVSVLLL	GETGVGKE	VIAHSVHLRSERAEQPFVAVNCA
DCTD	-LIGQTPVMENLRNIRLHIADTDVDVLVA	GETGSGKE	VVAQILHQVSHRRKGNFVALNCG
TYRR	QIVAVSPKMKHVVEQAQKLAMLSAPLLIT	GDTGTGKD	LFAYACHQASPRAGKPYLALNCA

FHLA	AMPAGLLESDFGHERGAFTGASAQIRGRFEL	ADKSSLFIDE	VGDMPLLELPKLLRLVQE
NIFA	ALPDNLLESELFGHEKGAFTGAVRQRKGRFEL	ADGGTLFIDE	IGESSASFQAKLLRIQE
FLBD	AIPENLLESELFGHEKGAFTGAMARRIGKFEE	ADGGTLFIDE	ISEMDVRLQAKLLRAIQE
NTRC	AIPKDLIESELFGHEKGAFTGANTIRQGRFEQ	ADGGTLFIDE	IGDMPLDVQTRLLRVLAD
XYLR	AIPPDLESELFGVDKGAFTGAVNARAGRFRER	ANGGTIFIDE	VIETPRAQATLLRLVQE
DCTD	ALPETVIESELFGHERGAFTGAQKRRTGRIEH	ASGGTLFIDE	IESMPAATQVKMLRVLEM
TYRR	SIPEDAVESELFGH-----APEGKKGFFEQ	ANGGSLFIDE	IGEMSPRMQAKLLRFLND

FHLA	QEFERLGSNKIIQTQDVRLIAATNRDLKKMVADREFRSDLYYRLNVFPIHLPLRERPEDIPLLA
NIFA	GEMERVGGDETLRVNVRIIAATNRHLEEEVRLGHFREDLYYRLNVMPIALPPLRERQEDIAELA
FLBD	REIDRVGGSKPVKNIRILATSNRDLAQAVKDGTFREDLLYRLNVNLRPLRERPADVISLC
NTRC	GOFYRVGGYAPVKVDVRIIAATHQNLEQRVQEGKFREDLFHRLNVIRVHLPLRERREDIPRLA
XYLR	GELERVGGDRTRKVDVRLITATNENLEEAVKMGFRADLFRLNVFPVHIPPLRERVEDIPLLV
DCTD	REITPLGTNEVRPVNLRVVAALKIDLGDPVAVRGDFREDLYYRLNVVTISIPPLRERRDDIPLLF
TYRR	GTFRRVGDEHVVHVDVRVICATQKNLVELVQKGMFREDLYYRLNVLTNLNPLRDCPQDIMPLT

FHLA	KAFTEFKIARRLGRNIDIPAETLRTLNSMEWPGNVRELENVIERAVLLTRG-NVLQL
NIFA	HFLVRKIAHSQGRTL-RISDGAIRLLMEYSWPGNVRELENCLESAVLSSES-GLIDR
FLBD	EFFVKKYSAANGIEEKPISAEAKRRLIAHRWPGNVRELENAHRAVLLSAG-PEIEE
NTRC	RHFLQVAARELGVEAKLLHPETEALTRLAWPGNVRLQLENTCRWLTVMAAGQEVLIQ
XYLR	EHFLRRHHKEYGKKTGLSDRAEACLHYQWPGNIRELENAERGVILTES-N--ES
DCTD	SHFAARAERFRDVPPLSPDVRRLASHTWPGNVRELSHVALRGVVEG-GGAAD
TYRR	ELFVARFADEQGVPRPKLAADLNTVLTRYAWPGNVRLKNAIYRA--LTQL-DGYEL

Figure 11. Alignment of the predicted "domain D" amino acid sequences of *E. coli* FHL-A, *K. pneumoniae* NIF-A, *C. crescentus* FLB-D, *E. coli* NTR-C, *P. putida* XYL-R, *R. leguminosarum* DCT-D, and *E. coli* TYR-R. Highlighted residues are identical or functionally similar to the FHL-A protein. Double underlined regions represent those residues of which five out of the six aligned amino acids are homologous to FHL-A protein. Boxed areas are the proposed ATP-binding regions.

polar mutations in "domain D" of NTR-C abolish the positive (not negative) control of the protein (147). Deletions in NIF-A suggest that only this central domain is necessary for transcriptional activation at the *nif* promoters (99). However, the other NIF-A domains are probably essential for specific regulation. Due to the high degree of homology, the central region of the FHL-A protein probably serves a similar function.

The final region IV (amino acids 663 to 682) of 20 amino acids is the suggested DNA binding domain (helix-turn-helix; ref. 39, 165) of the receiver proteins (58, 222). Both NTR-C and NIF-A have been shown to bind to upstream promoter sites by DNA footprinting (158, 179). This final segment of FHL-A protein is 50%, 45%, 40% and 30% homologous with the HYD-G, NTR-C, XYL-R and NIF-A proteins respectively (Fig. 12). Critical amino acids, Ala-5, Gly-9, Leu-15 and hydrophobic amino acids at positions 4, 8 and 10 (165) can be detected starting at position 663 (PKGAA QRLGL KRTTL LSRMK). This segment of the protein is crucial for the biological activity of the FHL-A protein. Inserting transposon Tn5 between the amino acids 662 and 663 (between the DNA bases G and G at positions 4404 and 4405 in plasmid pSE-133-2; Fig. 7) completely abolished the formate dependent expression of $\Phi(fhlB'-lacZ^+)$. Thus, the helix-turn-helix motif is necessary *in vivo* for specific activation of genes with FHL-A binding sites, potentially the UAS described by Birkmann and Böck (23). The *R. meliloti* NIF-A studies suggest that the C-terminal domain can be deleted without abolishing *in vivo* activation;

		1		4	5		8	9	10		15		18	20							
FHL-A	663	Pro	Lys	Gly	Ala	Ala	Gln	Arg	Leu	Gly	Leu	Lys	Arg	Thr	Thr	Leu	Leu	Ser	Arg	Met	Lys
HYD-G	417	Lys	Thr	Glu	Ala	Ala	Arg	Gln	Leu	Gly	Ile	Thr	Arg	Lys	Thr	Leu	Leu	Ala	Lys	Leu	Ser
NTR-C	444	Lys	Thr	Glu	Ala	Ala	Arg	Leu	Leu	Gly	Trp	Gly	Arg	Asn	Thr	Leu	Thr	Arg	Lys	Leu	Lys
XYL-R	534	Ile	Ser	Gln	Ala	Ala	Arg	Leu	Leu	Gly	Leu	Thr	Arg	Pro	Ala	Met	Ala	Tyr	Arg	Leu	Lys
NIF-A	496	Gln	Ala	Lys	Ala	Ala	Arg	Leu	Leu	Gly	Met	Thr	Pro	Arg	Gln	Val	Ala	Tyr	Arg	Ile	Gln



Figure 12. Alignment of potential DNA-binding motifs in the FHL-A, HYD-G, NTR-C, XYL-R and NIF-A proteins. Identical and functionally similar amino acids are highlighted. Critical amino acids for helix-turn-helix formation are indicated.

however, binding site specificity may be determined by this region, since *R. meliloti nifH* promoter activation was reduced 2-fold in *E. coli* and 10-fold in *R. meliloti* (99). Likewise, mutations in the helix-turn-helix region eliminate NTR-C repressive function in which binding to a specific operator sequence is required (147).

Primary structure of the *hydX'FB (hypC'DE)* genes upstream of the *fhIA* gene. Amino acid sequence data of the FHL-A protein suggests that a typical "sensor" protein (histidine-protein-kinase) is probably not required for FHL-A activity. However, the possibility of a nearby region encoding a sensor protein can not be ruled out. For example, the *K. pneumoniae nifLA* operon encodes both the activator (NIF-A) and its repressor (NIF-L). Sankar and Shanmugam (187, 188) described two genes, *hydF* and *hydB*, which were upstream of the *fhIA* gene. Based on genetic analysis, the 44 kDa and 32 kDa polypeptides coded by the *hydF* and *hydB* genes, respectively, appeared to interact with the *fhIA* gene product. Partial *hydF* gene products were capable of restoring total hydrogenase activity but only in the presence of multiple copies of *fhIA* gene (188). There appeared to be a direct correlation between the size of the overexpressed, partial HYD-F protein and the level of hydrogenase activity produced by the *hydF* mutants, strains SE-65 and SE-67. Additionally, the *hydB* gene appeared to substitute for the *hydF* gene in specific genetic backgrounds (SE-203 *hydF*, x^-). This suggested that *hydF*, *hydB*, *fhIA*, and an unidentified gene product could potentially interact in the production of FHL activity.

Therefore, the upstream DNA was sequenced in order to identify the characteristics of the *hydF* and *hydB* genes and their products. The sequence of a partial gene was also determined since it was present in the plasmid used for these experiments. Sequence analysis revealed that the *hyd* genes were transcribed in the same orientation as the *fhlA* gene (Fig. 9). The first open reading frame (bases 1 to 216) encodes the partial *hypC* product (3' end). The 72 amino acid residues identified have an anhydrous molecular weight of 7,848 Da. The second open reading frame, positions 219 to 1337, comprises the *hydF* gene. The gene codes for a protein of 373 amino acids with an anhydrous molecular weight of 41,363 Da which is comparable to the apparent molecular weight of 44,000 Da obtained by "maxicell" experiments (188). Positions 1379 to 2344 constitute the *hydB* gene which codes for a 322 amino acids product of an anhydrous molecular weight of 33,712 Da which again is comparable to the apparent molecular weight obtained by Sankar and Shanmugam (187). A typical "-10 and -35" or "-12 and -24" promoter sequence was not evident (between positions 1 to 2311); however, "Shine-Dalgarno" consensus sequences (positions 208 to 212 and 1367 to 1371) were located in front of both complete genes (*hydF* and *hydB*) sequenced. A 38 base pair intergenic region was located between the *hydF* and *hydB* genes. An inverted repeat (underlined in Fig. 9; positions 2387 to 2393 and positions 2397 to 2403) that can produce a 7-base-pairs stem and a 3-base (2394 to 2396) loop structure followed the *hydB* open reading frame. This region is not followed by a thymine residue stretch of significant

length, but the 17 base pairs region between this secondary structure and the start codon of the *fhlA* gene is 65% AT rich. Whether this is physiologically important has not been determined. The *hydB* gene product was extremely hydrophobic with a hydrophilic region in the amino-terminus. No significant homology of any *hyd* (*hyp*) genes sequenced could be found with known sequences in the GenBank or EMBL data bases and thus the potential mechanism by which these proteins participate in H_2 metabolism cannot be deduced.

Regulation of the *fhIA* Gene

Localization of an *fhIA* gene fusion. In order to study the regulation of *fhIA* gene, mutant strain SE-2007 was isolated as a *lac* operon fusion derivative of strain MC4100, using λ placMu53 (29). Upon detailed analysis, this strain was found to be defective in the production of dihydrogen. Plasmid complementation studies suggested that the fusion was located in the *fhIA* gene. The FHL activity of strain SE-2007 was restored by plasmids pSE-133 and pSE-133-1 (*hydB::Tn5*) but not by pSE-133-2 (*fhIA::Tn5*). Construction of a Hfr PO(*fhIA*) suggested the fusion mapped between the *srl* (58 min) and *cys* (59 min) genes; however, chromosomal transfer implied that direction of transcription was in the opposite orientation from that of the DNA sequence data. To eliminate the possibility of *fhIA* gene in high copy suppressing a mutation in a different gene in the 58 to 59 min region, restriction endonuclease digests of chromosomal DNA from strain SE-2007 were probed with an internal *SalI* to *PstI* fragment of the *fhIA* gene (2,946 to 4,269 bases; Fig. 9). Initial results with *ClaI* "digests" of chromosomal DNA from strain SE-2007 demonstrated that the *lac* fusion was within the *ClaI* fragment (positions 800 to 4601; Fig. 9); the hybridizing fragments were approximately 3.7 kb (strain MC4100) and 7.5 kb (strain SE-2007). Further analysis with *SalI*-*BglI*, *SalI*-*EcoRI*, and *SalI*-*ClaI* digests of chromosomal DNA verified that the fusion was within the *fhIA* gene between the *PstI* and *BglI* sites at the 3' end of

the open reading frame (positions 4,269 and 4,528 in Fig. 9; Fig. 13). This again reemphasizes the necessity of the carboxy-terminus for biological activity of the FHL-A protein.

Anaerobic induction of the *fhIA* gene. Regulation of expression of the *fhIA* gene was monitored by measuring the levels of β -galactosidase activity produced by strain SE-2007. When cultured under comparable aerobic conditions to mutant strain SE-2011, maximal dioxygen repression of *fhIA* gene expression was not obtained. Therefore, alternate procedures were implemented to maximally aerate the culture at low cell density (Table 7).

When cultured under microaerobic conditions, an overnight, stationary (1 ml in 13 x 100 mm tube with a metal-cap) culture of strain SE-2007 produced about 1,800 U of β -galactosidase activity (Table 7). Upon transfer to "strict" aerobic conditions (10 ml LB in 125 ml flask with 0.1 ml of overnight culture as inoculum; shaking at 250 rpm; 2 hr), utilized routinely for culturing other laboratory strains, the β -galactosidase activity of the culture decreased approximately 2-fold to about 1,000 units. Subsequent transfer of this culture to fresh LB and then aerobic growth for 1 hr further reduced the specific activity of the enzyme over 3-fold (290 U). This aerobic culture was used to inoculate LB and grown under anaerobic conditions. After 4 hr incubation, the specific activity of β -galactosidase of the culture was comparable to initial "microaerobic" values. These results indicate

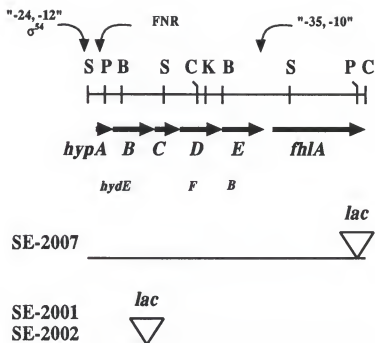


Figure 13. Localization of the promoter *lac* fusions in strains SE-2007 [Φ (*fhIA*'-'*lacZ*⁺)], SE-2001 [Φ (*hyp*'-'*lacZ*⁺)1], and SE-2002 [Φ (*hyp*'-'*lacZ*⁺)2]. The complete *hypABCDE* operon with the proposed σ^{54} - and FNR-dependent promoters has been redrawn with modifications from Lutz *et al.* (144). Previously identified *hydE*, *hydF* and *hydB* genes are noted (35, 187, 188, 238). Additionally, the weak "-35 and -10" constitutive promoter upstream of the *fhIA* gene, suggested in this study, is also indicated.

Table 7. Effect of dioxygen on the expression of $\Phi(fhlA'-'lacZ^+)$ in an *fhlA* mutant, strain SE-2007

Conditions	Innoculum size	Incubation time	β -Galactosidase Activity ^a
Microaerobic		16 hr	1,800
Aerobic-1	1%	2 hr	990
Aerobic-2	1%	1 hr	290
Anaerobic	10%	4 hr	1,800

^aExpressed as nanomoles of *o*-nitrophenol produced per minute per milligram of protein.

All cultures were grown at 37°C in Luria Broth.

An overnight stationary (1 ml in 13 x 100 mm metal-cap tube) LB culture of strain SE-2007 served as the inoculum for this experiment. This culture is referred above as the microaerobic culture. During the first part, 0.1 ml of this culture was inoculated into 10 ml LB (125 ml flask) and shaken at 250 rpm. After 2 hr of growth, 0.3 ml of this aerobic culture was transferred to 30 ml of fresh LB (1 L flask) and grown in the shaker for 1 hr. This culture was used to inoculate LB at 10% (v/v) and grown under anaerobic conditions. β -Galactosidase activities of all the cultures at the time of transfer to fresh medium (except the anaerobic culture, which was harvested after 4 hr growth) are presented in the Table.

that the *fhIA* gene is maximally induced at a higher oxygen tension in the growth medium than the *fhIB* gene.

Constitutive *fhIA* gene expression under anaerobiosis. Anaerobic expression of the *fhIA* gene was tested in LB medium supplemented with various effectors of the FHL pathway. Addition of alternate electron acceptors (nitrate and fumarate) did not influence the transcription of the *fhIA* gene (Table 8). Lower levels of β -galactosidase activity observed in cells grown in LB + nitrite medium may be a consequence of growth inhibition by nitrite. It is interesting to note that the exogenous formate and glucose did reduce the *fhIA* operon expression. LBG medium buffered with 0.1 M sodium phosphate to pH 7.0 reversed the effect of glucose on the levels of β -galactosidase produced by the culture. Additional experiments were done to verify that pH had no effect on *fhIA* gene expression using buffers ranging from pH 6.0 to 7.5 (0.1 M MES, pH 6.0; 0.1 M PIPES, pH 6.5; 0.1 M MOPS, pH 7.0; 0.1 M TES, pH 7.5). Again the results were comparable; low pH did not enhance *fhIA* gene transcription (data not shown).

Several genes known to be required for dihydrogen production, including *rpoN*, *narL*, *fnr*, *cya*, *molR*, *chlD* and *pfl*, were tested for their requirement in transcription of $\Phi(fhIA'-lacZ^+)$. Appropriate double-mutant strains were constructed to investigate the *fhIA* gene regulation. Analysis of these strains suggested that *fhIA* transcription is independent of known regulators of formate hydrogenlyase production (Table 9). The only double-mutant strain exhibiting reduced levels of

Table 8. Effect of media composition on the expression of $\Phi(fhlA^+ - lacZ^+)$ in an *fhlA* mutant, strain SE-2007

Medium	β -Galactosidase Activity ^a
Luria Broth	1,800
+ Formate (0.5%)	1,300
+ NO ₃ ⁻ (1.0%)	1,700
+ NO ₂ ⁻ (1.0%)	1,200
+ Glucose (0.3%)	1,200
+ Glucose + buffer ^b	2,000
+ Fumarate (0.5%)	1,700

All cultures were grown anaerobically at 37°C in Luria Broth ± supplements.

^aExpressed as nanomoles of *o*-nitrophenol produced per minute per milligram of protein.

^b0.1M Phosphate buffer at pH 7.0.

Table 9. Expression of $\Phi(fhlA'-'lacZ^+)$ in different genetic backgrounds

Strain	Relevant genotype	β -Galactosidase activity ^a
SE-2007	$\Phi(fhlA'-'lacZ^+)$	1,800
MJ-101	$\Phi(fhlA'-'lacZ^+) rpoN$	2,100
MJ-102	$\Phi(fhlA'-'lacZ^+) narL$	1,200
MJ-103	$\Phi(fhlA'-'lacZ^+) fnr$	1,600
MJ-107	$\Phi(fhlA'-'lacZ^+) cya$	800 ^b
MJ-108	$\Phi(fhlA'-'lacZ^+) molR$	1,400
MJ-109	$\Phi(fhlA'-'lacZ^+) pfl$	2,300
SE-2007	$\Phi(fhlA'-'lacZ^+)$	650/680 ^c
SE-1762	$\Phi(fhlA'-'lacZ^+) chlD$	830/800 ^c

All cultures were grown for 4 hr anaerobically at 37°C in Luria Broth unless otherwise indicated.

^aExpressed as nanomoles of *o*-nitrophenol produced per minute per milligram of protein.

^bPoor cell growth.

^cIndependent experiment in which cells were grown in LB/and LB supplemented with 1 mM Mo.

β -galactosidase activity is MJ-107, $\Phi(fhlA'-lacZ^+)$, *cya*. This 2-fold decrease in gene expression is probably due to the poor growth of the culture; however, a cAMP effect cannot be ruled out at this point.

Localization of *hyd* gene fusions. It has recently been suggested that the *fhlA* gene is regulated by the upstream σ^{54} - and FNR-dependent promoters of the *hyd* (*hyp*) operon (144; Fig. 13). In order to investigate this further, *hyd* gene fusions within this operon were studied to compare transcriptional regulation. Mutant strains SE-2001, SE-2002 and SE-2009 were similarly isolated as *lac* operon fusion derivatives of strain MC4100, using λ placMu53 (29). Biochemical analysis revealed that strains SE-2001 and SE-2002 were deficient in all three hydrogenase activities. Mutant strain SE-2009 was also significantly affected in total hydrogenase activity, however basal levels of hydrogenase were detectable. Therefore, these mutants were of interest in studying FHL regulation since they were phenotypically comparable to previously described *hyd* mutants.

Plasmid complementation analysis suggested that mutant strains SE-2001 and SE-2002 were located in the 58-59 min *hyd* cluster. Plasmid pSE-111 restored all three hydrogenase activities to parental levels in strains SE-2001 and SE-2002. Plasmid pSE-130 complemented only to approximately 35% of FHL activity in both mutants; whereas, HUP activity was restored to 20% and 100% in strains SE-2001 and SE-2002, respectively. Plasmids pSE-128 and pSE-125 did not complement the mutation in either strain SE-2001 or SE-2002. Evidently, the complete

hyd (*hyp*) operon (59 min) is required for restoration of hydrogenase activity in these fusion strains. Mutant strain SE-2009 was not complemented for hydrogenase activity with any of the plasmids tested. However, 250 μ M NiCl₂ supplementation of the growth medium restored FHL and HUP (H₂ to BV) activities to wild type level. Phage P1-transduction experiments confirmed that the *hyd* mutations in strains SE-2001 and SE-2002 were located between the *srl* (58 min) and *cys* (59 min) genes. However, the *lac* fusion in strain SE-2009 was not cotransducible within this region. Mutant strain SE-2009 is phenotypically comparable to strain HYD723, Φ (*hydC'*-'*lacZ*⁺), described by Wu *et al.* (239) which maps at 77.6 min in the *E. coli* chromosome.

DNA hybridization experiments localized *lac* gene fusions in strains SE-2001 and SE-2002 to a 0.9 kb *Bgl*I-*Sal*I fragment which contains the partial *hydE* (*hypB*) coding region (360 amino acids) and partial coding region of *hypC* (*hydX*) gene (20 amino acids) as determined by Lutz *et al.* (144; Fig. 13). Neither mutant was suppressed by nickel supplementation in the growth medium as in the case of point mutations in *hydE* gene. This could be explained by the polar effect of the fusions on the other *hyd* (*hyp*) genes whose products are also essential for production of active hydrogenase.

Genetic regulation of the *hyd* (*hyp*) operon. Regulation of expression of the *hyd* (*hyp*) operon was monitored by measuring the levels of β -galactosidase activity produced by strains SE-2001 and SE-2002. When cultured under strict aerobic conditions, both strains produced

about 20 U of β -galactosidase activity. Upon transfer to anaerobic conditions, *hyd* (*hyp*) operon expression was induced at a rate which paralleled the growth of the culture in LB medium. The specific activity of the enzyme reached maximum values of 170 U for SE-2001 and 190 U for SE-2002 (LB; Table 10). This peak value was reached during early stationary phase of growth (approximately 0.03 units $A_{420\text{nm}}$ or 10 μg cell protein) and remained constant throughout the remaining assay period (5 hr total). Thus, the increase in specific activity of the enzyme was coupled to growth (differential rate of induction approaching 1.0). In LB supplemented with glucose, formate or nitrate, the maximal β -galactosidase activity produced by strain SE-2001 was not significantly altered. The enzyme activity produced by strain SE-2002 also remained relatively constant in cells grown in LBG or LBN medium. In medium supplemented with 15 mM formate, the enhancing effect was only observed in strain SE-2002 in which the enzyme activity was slightly higher. These results are in direct contrast to the formate inducibility of the *fhlB* operon in which the levels of β -galactosidase activity were increased by 3.5-fold in the presence of 15 mM formate, and endogenously produced formate also had an enhancing effect.

To study the role of known effectors of dihydrogen metabolism, appropriate double mutants were constructed and β -galactosidase activity was monitored over a 5 hr time period. The only mutation which was consistent in its effect on *hyd* (*hyp*) operon expression was *fnr*. The *fnr* derivatives of strains SE-2001 and SE-2002 are strains SE-1651 and

Table 10. Anaerobic expression of $\Phi(hyp^{-}-'lacZ^{+})$, strains SE-2001 and SE-2002, in different genetic backgrounds and culture media

Strain	Relevant genotype	β -Galactosidase activity ^a			
		LB ^b	LBG ^b	LBF ^b	LBN ^b
SE-2001	$\Phi(hyp^{-}-'lacZ^{+})1$	170	170	210	150
SE-1654	$\Phi(hyp^{-}-'lacZ^{+})1 narL$	240	240	300	200
SE-1651	$\Phi(hyp^{-}-'lacZ^{+})1 fnr$	50	130	130	43
SE-1657	$\Phi(hyp^{-}-'lacZ^{+})1 rpoN$	53	135	160	130
SE-1658	$\Phi(hyp^{-}-'lacZ^{+})1 rpoN$	250	125	130	105
SE-1760	$\Phi(hyp^{-}-'lacZ^{+})1 chlD$	135/189 ^c	ND ^d	ND	ND
SE-2002	$\Phi(hyp^{-}-'lacZ^{+})2$	190	210	290	220
SE-1655	$\Phi(hyp^{-}-'lacZ^{+})2 narL$	100	145	160	95
SE-1652	$\Phi(hyp^{-}-'lacZ^{+})2 fnr$	22	140	120	50
SE-1660	$\Phi(hyp^{-}-'lacZ^{+})2 rpoN$	125	130	135	135
SE-1659	$\Phi(hyp^{-}-'lacZ^{+})2 rpoN$	140	135	150	130
SE-1761	$\Phi(hyp^{-}-'lacZ^{+})2 chlD$	135/202 ^c	ND	ND	ND

^aExpressed as nanomoles of *o*-nitrophenol produced per minute per milligram of protein

^bLB, Luria Broth; LBG, supplemented with 0.3% glucose; LBF, supplemented with 0.1% formate; LBN, supplemented with 0.1% nitrate.

^cLB grown culture/ LB supplemented with 1 mM Mo.

SE-1652, respectively. In LB with and without nitrate supplementation, the maximum β -galactosidase activity observed in these cultures was 50 U which is a 3- to 4-fold reduction in activity from the parent *hyd* (*hyp*) strains. Further analysis of these *fnr* double mutants, suggested that the FNR-dependent induction of the *hyd* (*hyp*) operon was alleviated upon formate or glucose supplementation (Table 10). When strains SE-1651 and SE-1652 were inoculated from microaerobic (1 ml standing LB cultures) to anaerobic conditions and cultured for 4 hr, the maximum activity of the cultures was comparable to activity in the presence of formate (data not shown). Perhaps the formate or FHL-A protein produced during the period of microaerobic conditions was sufficient for activation of the FNR-independent promoter(s) of the *hyd* (*hyp*) operon. The *rpoN* gene product was only required for anaerobic induction in one of the double mutants tested, strain SE-1657. The activity produced by this culture was reduced 3-fold from parental values, but only in LB medium. The *chlD* and *narL* mutations had no apparent effect on *hyd* (*hyp*) operon expression. Additionally, nickel supplementation (250 μ M) had no influence which is in contrast to the repressive effect in strain SE-2009, the *hyd* mutant which is not located in this 58-59 min region. It would be interesting to test whether a *hyd* (58 min) mutation, presumed to be necessary for nickel processing, would alleviate the nickel repression observed in strain SE-2009. Potentially, a protein-bound form of nickel would exert this negative effect on transcription.

These results are comparable to the DNA sequence data which

suggests multiple promoters for the *hyp* operon (FNR- and σ^{54} -dependent promoters (144). The FNR-dependent promoter within the *hypA* gene is apparently transcribed when formate concentrations are low as measured by β -galactosidase activity of strains SE-1651 and SE-1652 grown in LB medium. Formate supplementation alleviates the FNR requirement, suggesting transcription from FNR-independent promoter(s) in later stages of growth. The β -galactosidase produced by strain SE-1657 in LB medium, similarly, suggests a role for σ^{54} in *hyp* operon expression. Hybridization studies to *hyp* operon mRNA suggest that the *fhIA* gene is cotranscribed from the *hyp* promoters (A. Böck, personal communication). This is in contrast to the genetic regulatory pattern observed in strain SE-2007, $\Phi(fhIA'-'lacZ^+)$.

Two possibilities could mask the true physiological regulation of the *fhIA* gene and *hyd* (*hyp*) operon from the data presented above (strains SE-2007, SE-2001 and SE-2002). First, it is possible that the *fhIA* gene is autoregulated. The introduction of a gene fusion in the *fhIA* gene would inactivate its product, as seen phenotypically in strain SE-2007 (FHL activity). If FHL-A protein is required for regulation of *fhIA* gene transcription, the β -galactosidase values obtained for strain SE-2007 would not reflect this FHL-A requirement. Secondly, the *fhIA* gene could be transcribed from the *hyd* (*hyp*) promoter(s). The polarity of the *hyd* (*hyp*) mutations of strains SE-2001 and SE-2002 would inhibit *fhIA* gene transcription if *hyp* promoters were indeed the normal physiological promoters. Thus, if FHL-A is cotranscribed and is

required for formate inducibility of the *hyd* (*hyp*) operon, as is the case for *fhlB* operon expression, the increase would be absent in strains SE-2001 and SE-2002. Plasmid pSE-133, a *Cla*I insert retaining the partial *hydF* and complete *hydB*, *fhlA* genes, complements the *fhlA* mutants (SE-1174 and SE-2007). Even though the *hyd* (*hyp*) promoter(s) are absent in this plasmid, it is conceivable that the cloned genes are transcribed from a pBR-322 vector promoter.

Effect of multiple copies of the *fhlA* gene on *fhlA* and *hyd* (*hyp*) transcription. In order to study these possibilities, the effect of multiple copies of the *fhlA* gene on its own expression and transcription of the *hyd* (*hyp*) operon were monitored using plasmid pSE-133 (Fig. 14). The β -galactosidase activity of strains SE-2007 $\pm$ plasmid pSE-133 was comparable, irregardless of the presence of high copy *fhlA*⁺ or medium composition (Table 9). Interestingly, the *fhlA* gene is induced at very early stages of the organism's anaerobic growth to approximately 500 U. Then during late-log phase, a second induction occurs at a lower rate to a maximum value of about 1,500 U. The FHL-A protein would be available initially to activate the FHL pathway as formate is produced by the organism. In later stages of growth, formate levels would be higher (pH lower) and therefore, FHL-A protein would be necessary for maximum activation of the FHL system. However, transcription of the *fhlA* gene does not require formate as an inducer (Table 9).

From the data presented (Fig. 14), the *hyd* (*hyp*) operon expression differs from the *fhlA* gene regulation. In the presence of multiple

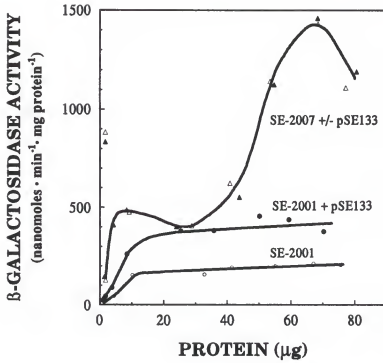


Figure 14. Differential rate of synthesis of $\Phi(fhlA'-lacZ^+)$, strain SE-2007, and $\Phi(hypX'-lacZ^+)$ 1, strain SE-2001, in LB medium supplemented with 3 mM formate in the presence and absence of the *fhlA*⁺ gene in a multicopy plasmid (pSE-133). β -Galactosidase activities and protein concentrations are expressed as units per ml and μ g per ml, respectively.

copies of *fhIA*⁺ gene (pSE-133) and formate (3 mM), the β -galactosidase activity produced by strain SE-2001 is increased by 2-fold. This would suggest that the *fhIA* gene is independent of the *hyd* (*hyp*) promoter(s) and is transcribed from its own "-35 and -10" σ^{70} promoter. The increased production of β -galactosidase activity in the SE-2001/pSE-133 culture is probably due to the unnaturally high concentration of FHL-A protein available in the cytoplasm. These results also suggest that the putative transcription termination region observed between the *hydB* and *fhIA* genes (Fig. 9) is physiologically significant.

Molybdate Metabolism and FHL ActivityCHL-D and MOL-R proteins are required for *fhlB* operon expression.

As was discussed earlier, both the *chlD* and *molR* gene products are necessary for *fhlB* operon expression (Table 6). Further analysis was done on the *chlD* and *molR* derivatives of strain SE-2011, strains SE-1714 and MJ-40 respectively, to better understand this regulation (Table 11). For these experiments, the strains were grown under anaerobic conditions in LB-formate medium, and the cells were harvested after 4 hr for enzyme assays. Under these conditions, a *molR* or *chlD* mutation reduced β -galactosidase activity produced by strain SE-2011 by over 4- to 5-fold. Supplementing the medium with 1 mM molybdate, restored enzyme levels to those of the parent, strain SE-2011. In the presence of multiple copies of the *fhlA* gene, the specific enzyme activity produced by strains MJ-40 and SE-1714 was increased by over 5-fold the activity of strains without plasmid pSE-133 (*fhlA*⁺). However, the enzyme levels of these cultures (+pSE-133) were approximately 2-fold less than the SE-2011/pSE-133 culture. Molybdate (1 mM) supplementation was still required for peak *fhlB* operon expression. These results suggest that the FHL-A mediated formate inducibility of the *fhlB* operon and molybdate-mediated regulation are separate and independent of each other.

Primary structure of the *molR* gene. Previous experiments identified the *molR* gene product as a putative regulatory element of both the *fdhF* and *ant* genes and essential for utilization of molybdate

Table 11. The effect of molybdenum on expression of $\Phi(fhlB^{-'}lacZ^{+})$ mutant, strain SE-2011 in *chlD* and *molR* genetic backgrounds

Strain	Relevant genotype	1mM Mo pSE133 ^b	β -Galactosidase Activity ^a			
			-	+	-	+
SE-2011	$\Phi(fhlB^{-'}lacZ^{+})$		700	700	1400	1500
MJ-40	$\Phi(fhlB^{-'}lacZ^{+})$ <i>molR</i>		130	640	720	1400
SE-1714	$\Phi(fhlB^{-'}lacZ^{+})$ <i>chlD</i>		160	700	900	1700

All cultures were anaerobically grown at 37°C in LB medium supplemented with 3 mM formate except SE-1714 which was grown in 15 mM formate.

^aExpressed as nanomoles of *o*-nitrophenol produced per minute per milligram of protein.

^bMulticopy plasmid carrying the *fhlA*⁺ gene

Values represent maximum activity reached during 4 hours of anaerobiosis.

by *E. coli* (134, 136). The physiological data presented above (Table 11) show that the MOL-R protein is needed for optimum induction of the *hhlB* operon. Therefore, the DNA sequence of the *molR* gene was determined to identify possible conserved motifs in the gene product (Fig. 15).

Plasmid pSE-1009 and exonuclease III generated deletion derivatives, plasmids 1 through 5 complemented a *molR* mutant (strain SE-1100) for FHL activity (plasmid derivatives 6 to 13 did not restore activity). Therefore, derivatives 5 to 13 were initially used for sequencing, since the complete *molR* gene was probably encoded by these plasmids (*ExoIII* derivatives are identified in Fig. 15). By searching GenBank and EMBL data bases with the preliminary sequence, it was determined that the *Sau3A*I-1,609-base-pair restriction fragment in pSJE301 which was cloned and sequenced by Johann and Hinton (110) is an internal fragment of the plasmid pSE-1009 sequence (2,927-base-pair *Kpn*I-*Eco*RV restriction fragment). Because the complete *chlJ*' and ORF3' DNA sequences have not yet been determined and the *molR* gene is upstream of the *chlD* gene, the region sequenced was extended to include the *Cla*I restriction site of plasmid pSE-1004 (for a total of 3,876-base-pair DNA sequenced).

Previous experiments have shown that the *chlD* expression is repressed by molybdate whereas the *molR* gene is constitutively transcribed (134, 155). This raises several unanswered questions. First if the *molR* gene product is regulatory, as the constitutive nature

CCCTGGCTAATGGAACCGGCAACGCAATGTGTGTTTTAGTAGCGAAATCGCGCGGGTGC	60
CGGAATAATTTTTGTTGGAGCTTAAGGGTGAGAAGGATTTTCGGCTGCATAACCAATGTCC	120
TGGCAAAAGTCTTATTGTGACGGAAAACGAACGCCACGCAAGCTGACCGCACAAAAGGG	180
GAGTGCTTTTCTGTGCTTAGCGGTAGAATAGTCTCATGACTATATCTGGAGTTGACCAT	240
<i>KpnI</i>	
GTTAGAGTTATTA AAAAGTCTGGTATTCGCCGTAATCAT <u>GGT</u> ACCTGTCGTGATGGCCAT	300
CATCCTGGGTCTGATTTACGGTCTTGGTGAAGTATTCACACTCTTTCTGGTGTGGTAA	360
<i>ExoIII</i> -1	
AAAAGACCAGCCCGGACAAAATCATTGATTCCTGAATGCCCGCTTAGTCGGGCATTTTC	420
<i>ExoIII</i> -2	
TTTTTCTCAACTTCTGCTTTTCTGCCGATATTTTTCTTATCTACCTCACAAAGGTTA	480
GCAATAACTGCTGGGAAAATCCGAGTTAGTCGTTATATTGTCGCTACATAACGTTACA	540
TTAAGGGGTACCAAT <u>GGT</u> CGTAAATGGTTGAACCTGTTTTCGGGGGGGGCACTCTCTT	600
TCGCTGTGCTGGCAATGCACTGGCAGATGAAGGGAAAATCACGGTGTTCGCCGCCGCAT	660
CACTGACTAACGCAATGCGAGCATCTTACGCAGTTAAAAAGAGAAAGCGTGGATG	720
<i>ExoIII</i> -3	
TGGTTTCTTCTTTCGCTTCGTCATCTACTCTCGCCCGTCAGATTGAAGCGGGTGC	780
CGGATCTGTTATTTCTGCCGATCAGAAATGGATGGATTATCGGTTGATAAAAAAGCGA	840
TCGATACAGTACGCGTCAGACACTGCTCGGCAATAGCCTGGTGTGTAGCACCGAAAG	900
CCAGCGTGCAAGAAAGATTCACCATCGACAGCAAAACCACTGGAATCTACTGCTGAATG	960
<i>ExoIII</i> -4	
GCGGTGCGCTGGCGGTTGGCGATCCGGAACATGTTCCCGTGGCATTATGCAAAAAGAG	1020
CACTGCAAAAACCTGGGCGCATGGGATACGCTCTCTCCGAACTGGCCCCAGCGGAAGATG	1080
TTCGTGGGCGCTGGCGCTGGTTCGAACGTAAACGAAGCGCTCTGGGCAATGTCTACGGT	1140
CTGACGCAGTTGCCAGCAAGGGGTTAAAGTGTTGCCACCTCCCGGAAGATTACATA	1200
AAAAAGTGAATATCCGGTGTCTGTTGTGGAAGGGCATAACAATGCCACAGCAAACTT	1260
<i>ExoIII</i> -5	
TTATGATTATCTGAAGGCACCGCAGGCACCCAAATCTTTAAACGTTACGGATTTACAATC	1320
<i>ExoIII</i> -6	
AAGTAATGATACTGACCGATCCAGAATGGCAGGCAGTTTATTAAGCCTGAAAGTTTCTT	1380
CCCTGGCTGTGCTGTTTAGCCTGCCGTTTGGGATCTTTTTGCCTGGTTACTGGTGCCTT	1440
GCACGTTTCCGGGCAAAAGCTGTGCTCGACAGCGTACTGCATCTACCGCTGGTGTACCGC	1500
<i>ExoIII</i> -7	
CCGTGGTGTGCGTTACTTATTATTAGTTTCGATGGGACGGCGGATTTATCGGTGAAC	1560
<i>ExoIII</i> -8	
GTCTGTATGACTGGTTGGTATTACCTTCGCTTTAGCTGGCGCGGCGGGTTCGCTG	1620
CCGCCGTATGTGCTTTCCGCTGATGGTGCGGGCAATTTCGCTGGCGCTGGAAGGGGTG	1680
ATGTCAAACCTGGAACAGCGCGCAAGAACTGGGGGCCGGCGCTGGCGCGTTTTCTTTA	1740
CTATCACGTTACCGTGACCTTACCGGAATTATTGTTGGTACGGTACTGGCTTTTGCTC	1800

Figure 15. Nucleic acid sequence of the *mol* (*chl*) operon. Restriction endonuclease sites for some of the enzymes are highlighted. Exonuclease III-generated deletion derivatives, plasmids #1 through 13 are identified. Translational start sites of ORF1 and ORF2 are double underlined (positions 555 to 557 and positions 1326 to 1328, respectively). "Shine-Dalgarno" sequence and translational start site of the *chlD* gene are single underlined (positions 2005 to 2009 and positions 2018 to 2020).

 ExoIII-9	
GTTCTCTCGGTGAGTTGGTGAACCATCACCTTTGTGTGCAACATTCCTGGTGAAACGC	1860
 ExoIII-10	
GAACCATTCCTTCTGCCATGTATACCCTGATCCAGACCCCGCGGGGAAAGTGGAGCGG	1920
CGAGACTGTGCATTATTTCTATTGCGCTGGCGATGATCTCCCTGTTGATTTGAGAATGGC	1980
TGGCCAGAATCAGCCGTGAACGGGCGGGCGCTAATCATGCTGGAATGAATTTTCCCA	2040
 ExoIII-11	
GACGTTGGGCAACCATTCCTGACTATTAATGAAACGCTGCCGCCAATGGCATCACTGC	2100
 ExoIII-12	
TATCTTTGGCGTCTCCGGTGCCGAAAAAATTTCGCTGATTAACGCCATCAGTGGACTGAC	2160
GCGCCCGCAAAAAGGGCGGATTGTCTCAATGGGCGGGTACTAAATGATGCCGAAAAAGG	2220
TATCTGCTGACGCGGAAAAAGCGTCGCGTTGGCTATGTTTTTCAGGATGCGCGGCTGTT	2280
 ExoIII-13	
CCCGCATTACAAAGTCGCTGGCAATCTGCGCTACGGCATGTGAAAAGTATGGTCGATCA	2340
GTTTCGATAAGCTGGTGGCGCTTTTAGGCATTGAACCGTTGCTTGACCGTTTACCAGGCGAG	2400
CCTGTCCGGAGGCGAAAAACAGCGCGTGGCGATTGGTCGGGCTTTGCTGACAGCACCGGA	2460
ATTGCTGTTGCTGGATGAACCGCTGGCGTCACTGGATATTCGCGTAAACGCGAACTGTT	2520
GCCTTATCTGCAACGGCTGACACGGGAAATCAACATTCGATGTTGTATGTGACGCCATTC	2580
CTGGATGAGATCTCCATCTGGCAGACAGAGTGGTACTGAAAACGGTCAGGTGAA	2640
AGCCTTTTGCCTGGAGGAAGTGTGGGGCAGTAGCGTGATGAATCCGTGGCTGCCGAAAGA	2700
GCAACAAAGTAGCATTCTGAAAGTGACGGTGCTTGAGCATCATCCGATTACGCGATGAC	2760
CGCGCTGGCGCTGGGCGATCAGCATTTGTGGGTCAATAAGCTGGACGAACCGCTGCAAGC	2820
TGCGCTACGTATCCGATTCAGGCTTCCGATGTTTCTCGGTTTTACAACCGCCGACGCA	2880
AACCCAGCATTCGTAACGTATTGCGGGCAAAAGTTGTTAATAGTTATGACGACAAACGGCCA	2940
GGTGAAGTGAACCTGGAAGTCGGCGGTAAAACGCTGTGGGCGCGTATCAGCCCGTGGGC	3000
CAGGGATGAACCTGGCGATCAAACTTGCCCTGTGGCTGTACGCGCAAATAAAAGTGTGTC	3060
GATAACCGCTGATTAATCAGGTGGCTATAAATGAACCTGGGCAATGCTGTGCGTGGTGT	3120
TATACCAATCACAATGTTGGCGCGCGCTTTTACCGGTATCGGCGTTGCCCATCGCCA	3180
EcoRV	
CGCCTGTACCAGCGGCTTCCAGCATACTGATATCATTAAGTATCGCCGAATGCCACGAC	3240
GTTCTCCATCGACCAACCTTGCCTCAACCCATTTTCGTCAAACGTTTACCTTTGCTGTT	3300
CGGCCCGGTGCAATATCAACCTGATCGTGCCAGGACCATTCACATCGCCCGCAGTTT	3360
ATGTTTCGACATGCTTACCAAAATGCTGCAATTGCCGACGGTATCGTGGCTCAGGGCGAA	3420
CTTCATACGCGGTTAACTTGTTCGCGCTTTCAGCCAGAGAAGCGACTTGTGTGAAAGT	3480
CGGACGCTGTTCCGGCGGCGAGGGTTTGCGCCAGTTAGATGTGCGAATGACATGCCCGGT	3540
CGGGTGCTCATAGACCATTTGCATCATCGACATACATCAGACCGTGAATGTGGTGTTTCATT	3600
CAGCCATCTCAATGAGTTGCAGGGCTTAAATACGGGCATTGGGTCCGCTTCCAGCACGG	3660
TTTTTGATGATAATCATACAAATAGGTGCCATTACAGCAAAATAGCAGGTGTATCCAGCG	3720
CCAGCGCTGATAAAAAGGATGAATAGCGACGTGATGGCGACCTGTGACGATGATTAATT	3780
ClaI	
GATAGCTGCTTCGCGAGCGCGGGCCAGGGCTTCTATCGATGAAGGAAGCAGGGTCTTTT	3840
TCGGGTCAATAAGGTGCCGTAAAGTCGAGAGCAA	3876

Figure 15—continued.

of the operon would suggest, it is possible that the operon it regulates when present on a high copy plasmid no longer requires MOL-R protein. Another possibility is that the *chlD* mutants synthesize a "molybdenum cofactor-binding protein" which binds MPT to form an active repressor (6, 14, 189). The *molR* mutant may be deficient in synthesizing the molybdenum-binding protein. Thus in the presence of molybdate, the repressor is absent and the *molR* gene is constitutively transcribed. Thirdly, the *mol* (*chl*) operon may be transcribed by multiple promoters. The *chlD* gene may be cotranscribed with the genes encoding the periplasmic-binding-protein dependent transport system for molybdate; whereas, the *molR* gene may be a regulatory protein transcribed from its own promoter. Further investigation will be required to answer these questions.

CONCLUSION

Genetic regulation of the FHL system encompasses a number of factors including anaerobiosis, nitrate, formate, low pH, molybdate, nickel and selenium. Genetic and physiological analysis of the *fhlB* operon can provide answers to the molecular mechanism by which these various components influence the levels of FHL activity in the cell. This study demonstrates that the *fhlB* operon is anaerobically inducible and requires σ^{54} , FHL-A, formate and molybdate for expression.

Sigma-54 is an alternate sigma factor required for *fhlB* operon expression and is encoded by the *rpoN* (*ntrA*, *glnF*) gene. By monitoring the expression of the *rpoN* gene using *lac* gene fusions, it has been demonstrated that this sigma factor is constitutively expressed in *E. coli*, *K. pneumoniae* and *R. meliloti* (34, 54, 154, 182). Whereas, the majority of alternate sigma factors which have been analyzed are inducible (as is the case for sporulation in *B. subtilis*; ref. 129, 142). This implies an interesting mechanism of transcriptional regulation at σ^{54} -dependent promoters. Currently it is presumed that transcriptional control is mediated predominately by modulation of the activator protein (receiver) and not by altering the levels of the σ^{54} -subunit (for review, see 90, 131). Therefore, identification and

analysis of the FHL activator is essential for full understanding of the genetic regulation of formate hydrogenlyase in *E. coli*.

This study shows that the *fhIA* gene encodes a putative transcriptional regulator which is homologous to other DNA-binding proteins of the two-component regulatory system. The "domain D" (presumed to be the site of ATP-binding and σ^{54} interaction) and helix-turn-helix motif are conserved; however, the amino-terminus of the FHL-A protein (first 353 amino acids) is unique among the various proteins analyzed. The FHL-A protein does not contain the conserved secondary structure or aspartate and lysine residues which have been shown to be phosphorylated by the respective histidine-protein-kinase (sensor). This suggests that FHL-A protein may be modulated by a mechanism differing from the typical phosphorylation cascade identified in the two-component systems.

Interestingly, the *fhIA* gene is induced by microaerobic conditions and constitutively synthesized anaerobically, while the *fhIB* gene (as well as *fdhF*, and *hyc* genes; ref. 171, 196) expression requires formate and molybdate in the medium (anaerobically). Transcription of the *fhIA* gene is apparently from the weak "-35 and -10" *fhIA* promoter (constitutive) and not from the FNR- or σ^{54} -dependent promoters of the *hyp* operon. Therefore, formate modulation of active FHL-A protein is not at the transcriptional level (this is independent of molybdate control; discussed below). Although recent data suggest that FHL-A alone can bind to URS of *fdhF* and retard electrophoretic mobility of

appropriate DNA, *in vivo* transcription assays have been unsuccessful to date (A. Böck, personal communication). This suggests that the FHL-A protein alone is a DNA-binding protein (however, purification of FHL-A as a formate complex cannot be ruled out at this time) but is not in an active form for transcriptional activation of the FHL pathway. Possibly another unidentified factor (formate and/or sensor protein) is required in the reaction.

From the *fhlB* gene regulation data presented in this study it can be deduced that formate is required for either (i) direct activation of FHL-A, (ii) transcription of another unidentified regulatory protein (sensor) which positively interacts with FHL-A, or (iii) direct interaction with a protein (sensor) which associates with FHL-A for transcription of FHL promoters. DNA sequence analysis of the nearby *hypC'DE* genes did not reveal a typical sensor protein (Fig. 9). Additionally, transcription of the *fdhF* gene in the absence of active FHL-B suggests that the *fhlB* gene is not this "unidentified sensor protein." Instead the FHL-B could potentially function as a modulator of the FHL pathway. Continued research will need to be done in this area to narrow the possibilities (i.e. formate binding studies with FHL-A protein, random mutagenesis of FHL-A protein to identify if FHL-A derivatives are formate independent, identification of a potential formate "sensor" protein).

The *fhlB* gene in *molR* or *chlD* genetic backgrounds still retained formate inducibility in the presence of multiple copies of the *fhlA*⁺

gene (exogenous molybdate was required for expression to wild type levels). This suggests that FHL is regulated by a dual regulatory system, one involving molybdate and a second formate-dependent pathway. DNA sequence analysis of the *mol (chl)* operon indicates that the *molR* gene, encoding a potential regulatory protein of the molybdate-dependent pathway (136) is located near genes encoding a periplasmic-binding-protein dependent molybdate transport system. The constitutivity of the *molR* gene suggests multiple promoters are involved in transcription of this operon.

These results show that fermentative dihydrogen production is regulated by a complex series of reactions involving the availability of formate, molybdate and the absence of oxygen. Further studies will be necessary in order to better understand the various mechanisms of regulation. Localization of the *fhlB* and *molR* mutations, primer extension analysis of the *mol (chl)* operon promoter region and identification of the parameters required for *in vitro* activation of the σ^{54} -dependent promoters of the FHL pathway will be beneficial to furthering our understanding of dihydrogen production in *E. coli*.

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BIOGRAPHICAL SKETCH

Julie Anne Maupin was born on July 30, 1963, in Richmond Heights, Missouri. She received a bachelor's degree in biological sciences in April 1985 and during that time received a honor society scholarship. Working in a biochemistry laboratory at the University of Alabama in Huntsville, she developed an enthusiasm for genetics and molecular biology. Since 1988, she has been in the Ph.D. program in the Department of Microbiology and Cell Science at the University of Florida.

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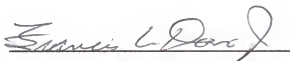
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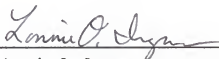
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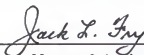
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December, 1991



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